PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 07 February 2000 (07.02.00)	
International application No. PCT/BE99/00089	**Applicant's or agent's file reference** P.SES.02/WO
International filing date (day/month/year) 09 July 1999 (09.07.99)	**Priority date** (day/month/year) 10 July 1998 (10.07.98)
Applicant LAUBER, E. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

27 December 1999 (27.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:
Juan Cruz

Telephone No.: (41-22) 338.83.38

WO 00/03025
PCT/BE99/00089

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:
VAN MALDEREN, Eric
Office Van Malderen
6 1, place Reine Fabiole
B-1083 Brussels
BELGIQUE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day month year) 20 January 2000 (20.01.00)	
Applicant's or agent's file reference P.SES.02/WO	**IMPORTANT NOTICE**

International application No.	**International filing date** (day month year)	**Priority date** (day month year)
PCT/BE99/00089	09 July 1999 (09.07.99)	10 July 1998 (10.07.98)

Applicant
SES EUROPE N.V./S.A. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,
   HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,
   SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   20 January 2000 (20.01.00) under No. WO 00 03025

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. 41-22/740.14.35

Authorized officer
J. Zahra

Telephone No. 41-22/338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
VAN MALDEREN, E
OFFICE VAN MALDEREN
Place Reine Fabiola 6/1
1083 Bruxelles
BELGIQUE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing (*day/month/year*): 25.01.00

Applicant's or agent's file reference: P.SES.02/WO

IMPORTANT NOTIFICATION

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/BE 99/00089	09/07/1999	10/07/1998

Applicant: SES EUROPE N.V./S.A. et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

27/12/1999

2. This date of receipt is:

- [X] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

[ ] (*If applicable*) This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA:
European Patent Office
D-80298 Munich
Tel. (- 49-89) 2399-0, Tx: 523656 epmu d
Fax: (- 49-89) 2399-4465

Authorized officer:
JAEGER D S
Tel. (- 49-89) 2399-2564

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, C07K 14/08, C12N 5/10, C12Q 1/68, A01H 5/00

A2

(11) International Publication Number: WO 00/03025

(43) International Publication Date: 20 January 2000 (20.01.00)

(21) International Application Number: PCT BE99 00089

(22) International Filing Date: 9 July 1999 (09.07.99)

(30) Priority Data:
98870159.5 10 July 1998 (10.07.98) EP

(71) Applicant *(for all designated States except US)*: SES EUROPE N.V./S.A. [BE/BE]; Industriepark 15, B-3300 Tienen (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LAUBER, E. [FR/FR]; 34, rue de Rotterdam, F-67000 Strasbourg (FR). GUILLEY, Hubert [FR/FR]; 32, rue de l'Herbe, F-67370 Berstett (FR). RICHARDS, Ken [FR/FR]; 2, rue Principale, F-67370 Pfulgriesheim (FR). JONARD, Gérard [FR/FR]; 9, quai de Chanoine Winterer, F-67000 Strasbourg (FR).

(74) Agents: VAN MALDEREN, Eric et al.; Office Van Malderen, 6/1, place Reine Fabiole, B-1083 Brussels (BE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD OF GENETIC MODIFICATION OF A WILD TYPE VIRAL SEQUENCE

(57) Abstract

The present invention concerns a method of genetic modification of a TGB-3 wild type viral sequence for reducing or suppressing the possible deleterious effects of the agronomic properties of a transformed plant or plant cell by said TGB-3 viral sequence, comprising the following successive steps: submitting said sequence to point mutation(s) which allow the substitution of at least one amino-acid into a different amino-acid; selecting genetically modified TGB-3 wild type viral sequences having said point mutation(s) and which are not able to promote cell-to-cell movement of a mutant virus having a dysfunctional TGB-3 wild type viral sequence, when expressed in trans from a replicon; further selecting among said genetically modified TGB-3 viral sequences, the specifically genetically modified sequence which inhibits infection with a co-inoculated wild type virus when the mutant form was expressed from a replicon; and recovering said specifically genetically modified TGB-3 viral sequence.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHOD OF GENETIC MODIFICATION OF A WILD TYPE VIRAL SEQUENCE

Field of the invention

The present invention is related to a method of genetic modification of a wild type viral sequence, for reducing or suppressing deleterious properties of plants or plant cells transformed by said wild type viral sequence.

The present invention is also related to the modified viral sequence obtained by said method, and to the plant and the plant cell comprising said modified viral sequence.

Background of the invention and state of the art

The widespread viral disease of the sugar beet plant (*Beta vulgaris*) called Rhizomania is caused by a furovirus, the beet necrotic yellow vein virus (BNYVV) (1, 2) which is transmitted to the root of the beet by the soilborne fungus *Polymyxa betae* (3).

The disease affects significantly acreages of the area where the sugar beet plant is grown for industrial use in Europe, USA and Japan and is still in extension in several places in Western Europe (4, 5).

Since 1986, number of reports and publications have described the use of isolated viral nucleotidic sequences expressed in plants to confer a high level of tolerance against a specific infectious virus or even to confer a broad spectrum type of resistance against a number of related viruses (6, 7, 8). One of the most documented viral resistance strategy based on genetic engineering, in many cultivated species such as potato, squash, cucumber or tomato, is the use of the viral nucleotidic sequence which under the control of plant regulatory elements, encodes the coat-protein of the target virus (9).

However, in coat-protein mediated resistance, the expression of a certain level of resistance in the transgenic plant might be attributed to different mechanisms such as RNA co-suppression and not necessarily to the production of the protein sequence.

In general, the virus sequence will be transformed in an appropriate cell or tissue culture of the plant species using an *Agrobacterium* mediated transformation system or a direct gene transfer method according to the constraints of the tissue culture or cell culture method which can be successfully applied in a given species. A whole plant will be regenerated and the expression of the transgene will be characterised.

Though sugar beet is known as a recalcitrant species in cell culture, limiting the extent of practical genetic engineering applications in that species, there are number of isolated reports of successful transformation and regeneration of whole plants (38). A few examples of engineering tolerance to the BNYVV by transforming and expressing the BNYVV coat-protein sequence in the sugar

beet genome have also been published (11, WO91/13159) though they rarely report data on whole functional transgenic sugar beet plants (12). In particular, reports show limited data on the level of resistance observed in infected conditions with transgenic sugar beet plants transformed with a gene encoding a BNYVV coat-protein sequence (13, 14).

A complete technology package including a sugar beet transformation method and the use of the expression of the BNYVV coat-protein sequence as resistance source in the transgenic sugar beet plant obtained by said transformation method has been described in the Patent Application WO91/13159.

Based on the information published, it can not be concluded that the coat-protein mediated resistance mechanism provides any potential for conferring to the sugar beet plant a total immunity to the BNYVV-infection by inhibiting completely the virus multiplication and diffusion mechanisms. To identify a resistance mechanism which significantly blocks the spread of the virus at the early stage of the infection process would be a major criteria of success to develop such a transgenic resistance. In addition, such resistance would diversify the mechanisms of resistance available.

Because the disease is shown to expand in many countries or areas, at a speed depending upon the combination of numerous local environmental and agricultural factors, there is a major interest to diversification and improvement of the genetic resistance mechanisms which may, alone or in combination, confer a stable and long lasting resistance strategy in the current

and future varieties of sugar beet plants which are grown for industrial use.

The genome of beet necrotic yellow vein furovirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4 and 5) are implicated in vector-mediated infection of sugar beet (*Beta vulgaris*) roots. Cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB), which encode, in order, the viral proteins P42, P13 and P15 (gene products are designated by their calculated M_r in kilodalton).

In the following description, the TGB genes and the corresponding proteins will be identified by the following terms : TGB-1, TGB-2, TGB-3 or by their encoded viral protein number P42, P13 and P15. TGB counterparts are present in other furoviruses and in potex-, carla- and hordeiviruses (15, 18, 19, 20, 21 and 22). In the enclosed table 1 are represented viruses having a TGB-3 sequence, the molecular weight of TGB-3 of said viruses, their host and references.

It has been shown previously that independent expression of P15 from a viral-RNA replication species known as a "replicon", derived from BNYVV RNA 3, inhibits infection with BNYVV by interfering cell-to-cell movement (16).

In order to introduce a virus comprising a TGB-3 nucleic acid sequence into a plant cell or a plant, it has been proposed to incorporate a nucleic acid construct comprising said TGB-3 nucleic acid sequence

operably linked to one or more regulatory sequences active in said plant (WO98/07875).

However, while expression of wild type TGB-3 viral sequence in a transgenic plant allows the blocking of said viral infection, the presence of said wild type sequence may induce deleterious effects on the agronomic properties of transformed plants or plant cells.

Aims of the invention

The present invention aims to provide a new method for inducing a genetic modification of a wild type viral sequence involved in the multiplication and diffusion mechanisms of virus infecting plants, in order to reduce or suppress the possible deleterious effects upon plants or plant cells transformed by said viral sequence.

Another aim of the present invention is to provide a method to obtain such a modified viral sequence which blocks virus infection when it is incorporated into a plant or a plant cell.

Summary of the invention

The present invention is related to a method of genetic modification of a TGB-3 wild type viral sequence, preferably the BNYVV P15 viral sequence, for reducing or suppressing the possible deleterious effects on the agronomic properties of the transformed plants or plant cells by said TGB-3 viral sequence.

Preferably, said genetic modification is a point mutation which allows the substitution of at least one amino-acid into another different amino-acid of said TGB-3 wild type sequence, preferably the substitution of at

least one amino-acid into another different amino-acid in the BNYVV P15 sequence.

It seems that the function of the TGB-3 wild type sequence in cell-to-cell movement involves at least in part "bridging" interactions between an element of the host plant (preferably a component of the plasmodesmata), and an element of viral origin (preferably another viral protein involved in cell-to-cell movement). Disruption of either the domain of the TGB-3 wild type sequence (which putatively interacts with the host element) or the domain of the TGB-3 wild type sequence (which putatively interacts with the viral element), allows the inhibition of the cell-to-cell movement.

In addition, it seems that said specific mutations in a TGB-3 wild type sequence allow the production of mutants produced in a transgenic plant, which will still interact with the viral element, but not with the host element. These mutants might compete for binding sites on the viral element of the TGB-3 wild type sequence produced in the initial stage of the viral infection, and abort the infection by inhibiting viral movement to an adjacent cell.

Advantageously, the substitution of at least one amino-acid into another different amino-acid of said sequence is made in regions rich in hydrophilic amino-acids usually present at the surface of the protein in its native configuration.

Preferably, the point mutation(s) allow the substitution of one or two amino-acids into one or two different amino-acids.

In the enclosed Table 1, preferred examples of said viruses having a TGB-3 wild type viral sequence,

the molecular weight of the corresponding TGB-3 peptide, their hosts and a reference, are described. The specific wild type P15 nucleotidic and amino-acid sequences of BNYVV are also already described (17).

The above-described point mutations were realised by conventional methods known by the person skilled in the art.

The above mutants containing the point mutation were tested for their ability to promote cell-to-cell movement of a viral mutant (with a dysfunctional TGB-3 sequence, preferably a BNYVV mutant with a dysfunctional P15 gene) when expressed in trans from a replicon. These mutants were incapable of promoting such movement and were tested for their ability to inhibit infection with a co-inoculated wild type TGB-3 virus, preferably co-inoculated with a wild type BNYVV, when the mutant form of the TGB-3 sequence, preferably the P15 gene, was expressed from a replicon.

The Inventors have discovered unexpectedly that the genetic modification method according to the invention (preferably a point mutation) could be used to obtain a modified TGB-3 viral sequence (preferably a modified BNYVV P15 sequence), which is able to block virus infection without producing deleterious effects when incorporated in the genome of a plant or a plant cell.

It is meant by "being able to block viral infection into a plant or a plant cell", the possibility to obtain a high degree of tolerance by the plant or plant cell transformed by said modified TGB-3 viral sequence to said viral infection, in particular the possibility to ensure rapid and total blocking of the virus multiplication and diffusion mechanisms into the plant, preferably the

blocking of the BNYVV virus multiplication and diffusion mechanisms into a sugar beet plant (beta vulgaris), including fodder beet, Swiss Whard and table beet which may also be subjected to said BNYVV infection.

Said tolerance or resistance could be easily measured by various methods well known by the person skilled in the art.

Preferably, the genetic modifications in the TGB-3 wild type viral sequence are point mutations in the portions of said wild type viral sequence involved in the mechanisms of viral cell-to-cell movements.

The present invention is also related to the modified TGB-3 viral nucleotidic and amino-acid sequences obtained (recovered) by said (modification and selection) method, more preferably the BNYVV P15 modified nucleotidic and amino-acid sequences obtained (recovered) by said method.

Preferably, said BNYVV P15 nucleotidic and amino-acid sequences are selected from the group consisting of the following nucleotidic or corresponding amino-acid sequences :

SEQ ID NO 1 :

ATGGTGCTTGTGGTTGCAGTAGCTTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M V L V V A V A L S N I V L Y I V A G C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V V V S M L Y S P F F S N D V K A S S Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGACAGGAATTCGTTTGCTCAATTT 180
A G A I F K G S G C I M D R N S F A Q F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G S C D I P K H V A E S I T K V A T K E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H D V D I M V K R G E V T V R V V T L T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGCGGTGTTTTTGTTCATGATA 360
E T I F I I L S R L F G L A V F L F M I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C L M S I V W F W Y H R *

SEQ ID NO 2 :

ATGGTGCTTGTGGTTAAAGTAGATTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M V L V V K V D L S N I V L Y I V A G C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V V V S M L Y S P F F S N D V K A S S Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGCCGCGAATTCGTTTGCTCAATTT 180
A G A I F K G S G C I M A A N S F A Q F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G S C D I P K H V A E S I T K V A T K E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H D V D I M V K R G E V T V R V V T L T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGCGGTGTTTTTGTTCATGATA 360
E T I F I I L S R L F G L A V F L F M I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C L M S I V W F W Y H R *

SEQ ID NO 3 :

```
ATGGTGCTTGTGGTTAAAGTAGATTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M  V  L  V  V  K  V  D  L  S  N  I  V  L  Y  I  V  A  G  C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V  V  V  S  M  L  Y  S  P  F  F  S  N  D  V  K  A  S  S  Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGACAGGAATTCGTTTGCTCAATTT 180
A  G  A  I  F  K  G  S  G  C  I  M  D  R  N  S  F  A  Q  F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G  S  C  D  I  P  K  H  V  A  E  S  I  T  K  V  A  T  K  E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H  D  V  D  I  M  V  K  R  G  E  V  T  V  R  V  V  T  L  T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGATGATTTTTTGTTCATGATA 360
E  T  I  F  I  I  L  S  R  L  F  G  L  D  D  F  L  F  M  I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C  L  M  S  I  V  W  F  W  Y  H  R  *
```

In the following description, the various modified BNYVV TGB-3 sequences will be hereafter called "P15 mutants", identified by the following reference : BNP15-Ala1, corresponding to SEQ ID NO 1, BNP15-Ala4 corresponding to SEQ ID NO 2, BNP15-Asp9, corresponding to SEQ ID NO 3.

The nucleotidic and corresponding amino-acid sequences of SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3 can be compared to SEQ ID NO 4, which is the sequence of the wild type P15 nucleotidic and amino-acid sequence already described (17).

The present invention is also related to the vector comprising said modified nucleotidic sequence possibly being operably linked to one or more regulatory sequence(s) active into a plant or a plant cell. Preferably, said vector is a plasmid comprising already said regulatory sequence(s) active into a plant or a plant cell.

The present invention is also related to a method for inducing a resistance to a virus comprising a TGB-3 sequence, preferably one of the viruses described in the enclosed Table 1, and more preferably the BNYVV virus, said method comprising the following steps :

- preparing a nucleic acid construct comprising a nucleic acid sequence being genetically modified according to the method of the invention and being operably linked to one or more regulatory sequences active into a plant or a plant cell,
- transforming the plant cell with the nucleic acid construct, and
- possibly regenerating the transgenic plant from the transformed plant cell.

Preferably, said method is used for inducing a resistance to the BNYVV into a sugar beet plant or a sugar beet cell. Said method comprises the following steps :

- preparing a nucleic acid construct comprising a modified nucleic acid sequence obtained by the method according to the invention, preferably preparing a nucleic acid construct comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2 or

SEQ ID NO 3, being operably linked to one or more regulatory sequences active into a plant,

- transforming the sugar beet plant cell with the nucleic acid construct, and
- possibly regenerating the transgenic sugar beet plant from the transformed sugar beet plant cell.

The present invention is also related to the obtained (recovered) transgenic plant or the transgenic plant cell resistant to an infection by a virus comprising a TGB-3 sequence, preferably one of the viruses described in the enclosed Table 1, more preferably the BNYVV virus, said plant or plant cell comprising a nucleic acid construct having a TGB-3 modified nucleic acid sequence, being operably linked to one or more regulatory sequences capable of being active into a plant or a plant cell.

Preferably, said modified nucleic acid sequence is selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3, being operably linked to one or more regulatory sequences active into a plant or a plant cell.

Preferably, the cell is a stomatal cell and the regulatory sequence comprises a promoter sequence and a terminator sequence capable of being active into a plant. Said promoter sequence can be constitutive or could be obtained from a foreigner promoter sequence, and is preferably selected from the group consisting of the 35S Cauliflower Mosaic Virus promoter, and/or the polyubiquitin Arabidopsis thaliana promoter.

Advantageously, the promoter sequence is a promoter which is mainly capable of being active in the root tissue of plants such as the par promoter or the haemoglobin gene from Perosponia andersonii.

A last aspect of the present invention is related to a transgenic plant tissue such as fruit, stem, root, tuber, seed of the transgenic plant according to the invention or a reproducible structure (preferably selected from the group consisting of calluses, buds or embryos) obtained from the transgenic plant or the plant cell according to the invention.

The techniques of plant transformation, tissue culture and regeneration used in the method according to the invention are the ones well known by the person skilled in the art. Such techniques are preferably the ones described in the International Patent Applications WO95/101778, WO91/13159 (corresponding to the European Patent Application EP-B-0517833), WO98/07875, which are incorporated herein by reference.

These techniques are preferably used for the preparation of transgenic sugar beet plants and plant cells according to the invention.

REFERENCES

1. Tamada T. & Baba T., *Annals of the Phytopathological Society of Japan* **39**, pp. 325-332 (1973)
2. Kuszala M.& Putz C., *Annals of Phytopathology* **9**, pp. 435-446 (1977)
3. Keskin B., *Archiv für Mikrobiology* **49**, pp. 348-374 (1964)
4. Asher M.J.C., *Rhizomania* In *The sugar beet crop*, ed. D.A. Cooke and R.K. Scott, Chapman & Hall, London, pp. 312-338 (1993)
5. Richard-Molard M., *Rhizomanie* In *Institut français de la betterave industrielle. Compte-rendu des travaux effectués en* 1994, ITB, Paris pp. 225-229 (1995)
6. Powell A.P. et al., *Science* **232**, pp. 738-743 (1986)
7. Fritchen J.H. & Beachy R.N., *Ann. Rev. Microbiol.* **47**, pp. 739-763 (1993)
8. Wilson T.M.A., *Proc. Natl. Acad. Sci. USA* **90**, pp. 3134-3141 (1993)
9. Gonsalves D. & Slightom J.L., *Seminars in Virology* **4**, pp. 397-405 (1993)
10. D'Halluin K. et al., *Biotechnology* **10**, pp. 309-314 (1992)
11. Kallerhof J. et al., *Plant Cell Reports 9*, pp. 224-228 (1990)
12. Ehlers U. et al., *Theoretical and Applied Genetic* **81**, pp. 777-782 (1991)
13. Kraus J. et al., *Field performance of transgenic sugar beet plants expresing BNYVV coat protein plants, Fourth International Congress of Plant Molecular Biology, Int. Soc. for Plant Molecular Biology*, Amsterdam (1994)
14. Maiss E. et al., *Proceedings of the Third International Symposium on the Biosafety Results of Field Tests of*

Genetically Modified Plants and Microorganisms, Monterey, pp. 129-139 (1994)

15. Gilmer et al., *Virology* **189**, pp. 40-47 (1992)
16. Bleykasten-Grosshans et al., *Mol. Plant-Microbe Interact.* **10**, pp. 240-246 (1997)
17. Bouzoubaa et al., *J. Gen. Virol.* **67**, pp. 1689-1700 (1986)
18. Richards & Tamada, *Annu. Revendication. Phytopathol.* **30**, pp. 291-313 (1992)
19. Bouzoubaa et al., *J. Gen. Virol.* **68**, pp. 615-626 (1987)
20. Herzog et al., *J. Gen. Virol.* **18**, pp. 3147-3155 (1994)
21. Scott et al., *J. Gen. Virol.* **75**, pp. 3561-3568 (1994)
22. Koonin & Dolja, *Crit. Revendication. Biochem. and Mol. Biol.* **28**, pp. 375-430 (1993)

Table 1

Virus	Size of TGB-3	Host	Reference
Apple stem pitting virus	8 kDa	apple	Jelkman, *J. Gen. Virol.* 75, 1535-1542 (1994)
Blueberry scorch virus	7 kDa	blueberry	Cavileer et al., *J. Gen. Virol.* 75, 711-720 (1994)
Potato virus M	7 kDa	potato	Zavriev et al., *J. Gen. Virol.* 72, 9-14 (1991)
White clover mosaic virus	8 kDa	clover	Forster et al., *Nucl. Acids Res.* 16, 291-303 (1988)
Cymbidium mosaic virus	10 kDa	orchid	Neo et al., *Plant Mol. Biol.* 18, 1027-1029 (1992)
Potato virus X	8 kDa	potato	Rupasov et al., *J. Gen. Virol.* 70, 1861-1869 (1994)
Barley stripe mosaic virus	17 kDa	barley	Gustafson et al., *Nucl. Acids Res.* 14, 3895-3909 (1986)
Potato mop top virus	21 kDa	potato	Scott et al., *J. Gen. Virol.* 75, 3561-3568 (1994)
Peanut clump virus	17 kDa	peanut	Herzog et al., *J. Gen. Virol.* 75, 3147-3155 (1994)
Beet soil-borne virus	22 kDa	Sugar beet	Koenig et al., *Virology* 216, 202-207 (1996)

PATENT COOPERATION TREATY

RECD 07 DEC 2000

15

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P.SES.02/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/BE99/00089	09/07/1999	10/07/1998
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant SES EUROPE N.V./S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27/12/1999	05.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Claes, B Telephone No. +49 89 2399 8429

Form PCT IPEA 409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/BE99/00089

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-22 as originally filed

Claims, No.:

1-22 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-20
	No:	Claims	21,22
Inventive step (IS)	Yes:	Claims	1-20
	No:	Claims	21,22
Industrial applicability (IA)	Yes:	Claims	1-22
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The following documents are referred to:
   D1 = WO98/07875
   D2 = Beck et al. (1994), PNAS USA, 91, 10310-10314.
   D3 = Seppänen et al. (1997), J. Gen. Virol., 78, 1241-1246.

2. D1 teaches that overexpression of the TGB-3 gene in a tobacco disturbs the TGB-2/TGB-3 ratio necessary for the movement of BNYVV in plants (see e.g. p.26-27 and claim 1). Consequently, the transgenic expression of TGB-3 resulted in BNYVV resistance.
   TGB-1, TGB-2 and TGB-3 are known to be viral genes involved in viral cell-to-cell movement.

   D2 discloses that plant expression of a mutated 13kd TGB-2 gene of White clover Mosaic virus strain O (a species of the potexvirus genus) results in resistance to systemic infection with a whole array of Potexviri (see e.g. D1 abstract). Similar results were obtained in D3 for potato virus X mutant genes.

3. D1 does not describe or suggest any specific mutation in TGB-3 wild type viral sequences. The only mutated TGB genetic sequences are made in other TGB sequences than the TGB-3 sequence. Overexpression of TGB-2 genes, according to the applicant, will not confer resistance to aplant. Therefore, it was not obvious for the person skilled in the art to combine the teaching of D1 with that of D2 and/or D3 in order to arrive at the claimed invention. Accordingly an inventive step can be acknowledged (Article 33(3) PCT).

4. Claims 21 and 22 also embrace known products. More in particular claim 21 does not require the transgenic trait to be related to the TGB-3 gene, nor does it require the transgenic plant of claim 14 to comprise the mutant TGB-3 gene in every cell. The seed ("reproducible structure") of a transgenic plant does not necessarily contain the transgenic trait (hemizygosity).
   Consequently, the subject matter of claims 21 and 22 lacks novelty over known

subject matter (Article 33(3) PCT).

In this context it is noted that under the PCT, the IPEA is not allowed to execute any amendmends suggested by the Applicant.

5. D1 discloses a truncated P15 gene of BNYVV which is capable of interfering with the cell-to-cell movement activity of wild type P15 (see e.g. D1 paragraph bridging p.26 and 27).

Applicant shown that the TGB-3 constructs as disclosed in D1 do not block efficiently the cell-to-cell movement as obtained with the specific mutated genetic sequence accrording to the invention. Consequently, in view of the feature "which are not able to promote cell-to-cell movement" in claim 1 the claimed subject matter is novel as required under Article 33(2) PCT.

Re Item VIII

Certain observations on the international application

1. The use of the wording "foreigner" in the context of claim 11 for defining a promoter seems not to conform conventional nomenclature current in the technical field (Article 6).

2. The wording "TGB-3 wild type viral sequence" lacks clarity as it is open ended and hence open to interpretation.

3. The sequence IDs as mntionned in claim 4 are not in line with the sequence IDs as represented in the sequence listing

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA 220) as well as, where applicable, item 5 below	
International application No	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/BE 99/00089	09/07/1999	10/07/1998

Applicant

SES EUROPE N.V./S.A. et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1 **Basis of the report**

a With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2 [X] **Certain claims were found unsearchable** (See Box I).

3 [ ] **Unity of invention is lacking** (see Box II).

4 With regard to the **title**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5 With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6 The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures

INTERNATIONAL SEARCH REPORT

International application No
PCT/BE 99/00089

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: -
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

JAN 10 2001 PATENT OFFICE

International Application No
PCT/BE 99/00089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/08 C12N5/10 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994 (1994-10), pages 10310-10314, XP002025561 especially abstract the whole document ---	1-7,9
Y	WO 98 07875 A (SES EUROP N V S A ;LEFEBVRE MARC (BE); WEYENS GUY (BE); BLEYKASTEN) 26 February 1998 (1998-02-26) cited in the application pages 6,9,14; page 15, line 9; page 23,25,28,29; claims ---	1-7,9
	-/--	

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

* Special categories of cited documents

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 1999	25/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. +31-70 340-2040 Tx. 31 651 epo nl Fax +31-70 340-3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 99/00089

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GILMER D ET AL: "EFFICIENT CELL-TO-CELL MOVEMENT OF BEET NECROTIC YELLOW VEIN VIRUS REQUIRES 3' PROXIMAL GENES LOCATED ON RNA 2" VIROLOGY, vol. 189, 1992, pages 40-47, XP002025562 ---	1-22
A	XU H ET AL: "GENETICALLY ENGINEERED RESISTANCE TO POTATO VIRUS X IN FOUR COMMERCIAL POTATO CULTIVARS" PLANT CELL REPORTS, vol. 15, 1995, pages 91-96, XP000617900 ---	1-22
A	SEPPAENEN P ET AL: "MOVEMENT PROTEIN-DERIVED RESISTANCE TO TRIPLE GENE BLOCK -CONTAINING PLANT VIRUSES" JOURNAL OF GENERAL VIROLOGY, vol. 78, no. PART 06, June 1997 (1997-06), pages 1241-1246, XP002073446 the whole document ---	1-22
A	WO 91 13159 A (BIOSEM) 5 September 1991 (1991-09-05) cited in the application pages 1,2,16,19; examples , claims the whole document -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 99/00089

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807875 A	26-02-1998	AU 3935097 A	06-03-1998
		EP 0938574 A	01-09-1999
WO 9113159 A	05-09-1991	FR 2658987 A	06-09-1991
		FR 2658988 A	06-09-1991
		AT 129745 T	15-11-1995
		DE 69114275 D	07-12-1995
		DE 69114275 T	13-06-1996
		DK 517833 T	04-12-1995
		EP 0517833 A	16-12-1992
		ES 2079647 T	16-01-1996

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 January 2000 (20.01.2000)

PCT

(10) International Publication Number
WO 00/03025 A3

(51) International Patent Classification[6]: C12N 15/82, C07K 14/08, C12N 5/10, C12Q 1/68, A01H 5/00

(21) International Application Number: PCT/BE99/00089

(22) International Filing Date: 9 July 1999 (09.07.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
98870159.5 10 July 1998 (10.07.1998) EP

(71) Applicant *(for all designated States except US)*: **SES EUROPE N.V./S.A.** [BE/BE]; Industriepark 15, B-3300 Tienen (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LAUBER, E.** [FR/FR]; 34, rue de Rotterdam, F-67000 Strasbourg (FR). **GUILLEY, Hubert** [FR/FR]; 32, rue de l'Herbe, F-67370 Berstett (FR). **RICHARDS, Ken** [FR/FR]; 2, rue Principale, F-67370 Pfulgriesheim (FR). **JONARD, Gérard** [FR/FR]; 9, quai de Chanoine Winterer, F-67000 Strasbourg (FR).

(74) Agents: **VAN MALDEREN, Eric** et al.; Office Van Malderen, 6/1, place Reine Fabiole, B-1083 Brussels (BE).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
16 August 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/03025 A3

(54) Title: METHOD OF GENETIC MODIFICATION OF A WILD TYPE VIRAL SEQUENCE

(57) Abstract: The present invention concerns a method of genetic modification of a TGB-3 wild type viral sequence for reducing or suppressing the possible deleterious effects of the agronomic properties of a transformed plant or plant cell by said TGB-3 viral sequence, comprising the following successive steps: submitting said sequence to point mutation(s) which allow the substitution of at least one amino-acid into a different amino-acid; selecting genetically modified TGB-3 wild type viral sequences having said point mutation(s) and which are not able to promote cell-to-cell movement of a mutant virus having a dysfunctional TGB-3 wild type viral sequence, when expressed in trans from a replicon; further selecting among said genetically modified TGB-3 viral sequences, the specifically genetically modified sequence which inhibits infection with a co-inoculated wild type virus when the mutant form was expressed from a replicon; and recovering said specifically genetically modified TGB-3 viral sequence.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 99/00089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/08 C12N5/10 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994 (1994-10), pages 10310-10314, XP002025561 especially abstract the whole document	1-7,9
Y	WO 98 07875 A (SES EUROP N V S A ;LEFEBVRE MARC (BE); WEYENS GUY (BE); BLEYKASTEN) 26 February 1998 (1998-02-26) cited in the application pages 6,9,14; page 15, line 9; page 23,25,28,29; claims	1-7,9

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 1999	25/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GILMER D ET AL: "EFFICIENT CELL-TO-CELL MOVEMENT OF BEET NECROTIC YELLOW VEIN VIRUS REQUIRES 3' PROXIMAL GENES LOCATED ON RNA 2" VIROLOGY, vol. 189, 1992, pages 40-47, XP002025562 ---	1-22
A	XU H ET AL: "GENETICALLY ENGINEERED RESISTANCE TO POTATO VIRUS X IN FOUR COMMERCIAL POTATO CULTIVARS" PLANT CELL REPORTS, vol. 15, 1995, pages 91-96, XP000617900 ---	1-22
A	SEPPAENEN P ET AL: "MOVEMENT PROTEIN-DERIVED RESISTANCE TO TRIPLE GENE BLOCK -CONTAINING PLANT VIRUSES" JOURNAL OF GENERAL VIROLOGY, vol. 78, no. PART 06, June 1997 (1997-06), pages 1241-1246, XP002073446 the whole document ---	1-22
A	WO 91 13159 A (BIOSEM) 5 September 1991 (1991-09-05) cited in the application pages 1,2,16,19; examples , claims the whole document -----	1-22

INTERNATIONAL SEARCH REPORT

national application No.
PCT/BE 99/00089

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: -
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 99/00089

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807875 A	26-02-1998	AU 3935097 A	06-03-1998
		EP 0938574 A	01-09-1999
WO 9113159 A	05-09-1991	FR 2658987 A	06-09-1991
		FR 2658988 A	06-09-1991
		AT 129745 T	15-11-1995
		DE 69114275 D	07-12-1995
		DE 69114275 T	13-06-1996
		DK 517833 T	04-12-1995
		EP 0517833 A	16-12-1992
		ES 2079647 T	16-01-1996

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 1.2

The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 99/00089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/08 C12N5/10 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BECK D L ET AL: "DISRUPTION OF VIRUS MOVEMENT CONFERS BROAD-SPECTRUM RESISTANCE AGAINST SYSTEMIC INFECTION BY PLANT VIRUSES WITH A TRIPLE GENE BLOCK" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994 (1994-10), pages 10310-10314, XP002025561 especially abstract the whole document ---	1-7,9
Y	WO 98 07875 A (SES EUROP N V S A ;LEFEBVRE MARC (BE); WEYENS GUY (BE); BLEYKASTEN) 26 February 1998 (1998-02-26) cited in the application pages 6,9,14; page 15, line 9; page 23,25,28,29; claims ---	1-7,9

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 1999	25/10/1999

Name and mailing address of the ISA
European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl
Fax (+31-70) 340-3016

Authorized officer
Holtorf, S

1

Form PCT ISA 210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BE 99/00089

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: -
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The SEQIDs as mentioned in claim 4 do not fall in line with the SEQIDs as represented in the sequence listings.

3. ☐ Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest

☐ No protest accompanied the payment of additional search fees.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GILMER D ET AL: "EFFICIENT CELL-TO-CELL MOVEMENT OF BEET NECROTIC YELLOW VEIN VIRUS REQUIRES 3' PROXIMAL GENES LOCATED ON RNA 2" VIROLOGY, vol. 189, 1992, pages 40-47, XP002025562 ---	1-22
A	XU H ET AL: "GENETICALLY ENGINEERED RESISTANCE TO POTATO VIRUS X IN FOUR COMMERCIAL POTATO CULTIVARS" PLANT CELL REPORTS, vol. 15, 1995, pages 91-96, XP000617900 ---	1-22
A	SEPPAENEN P ET AL: "MOVEMENT PROTEIN-DERIVED RESISTANCE TO TRIPLE GENE BLOCK -CONTAINING PLANT VIRUSES" JOURNAL OF GENERAL VIROLOGY, vol. 78, no. PART 06, June 1997 (1997-06), pages 1241-1246, XP002073446 the whole document ---	1-22
A	WO 91 13159 A (BIOSEM) 5 September 1991 (1991-09-05) cited in the application pages 1,2,16,19; examples , claims the whole document -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 99/00089

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807875 A	26-02-1998	AU 3935097 A	06-03-1998
		EP 0938574 A	01-09-1999
WO 9113159 A	05-09-1991	FR 2658987 A	06-09-1991
		FR 2658988 A	06-09-1991
		AT 129745 T	15-11-1995
		DE 69114275 D	07-12-1995
		DE 69114275 T	13-06-1996
		DK 517833 T	04-12-1995
		EP 0517833 A	16-12-1992
		ES 2079647 T	16-01-1996

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below	
P.SES.02/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/BE 99/00089	09/07/1999	10/07/1998
Applicant		
SES EUROPE N.V./S.A. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[X] as suggested by the applicant.

[ ] None of the figures.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention

Plant Biology

PD 10-94
P 10310-10314 = (5)

XP 002025561

Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block

transgenic plant / dominant negative mutation / dysfunctional movement protein

DAVID L. BECK, CRAIG J. VAN DOLLEWEERD, TONY J. LOUGH, EZEQUIEL BALMORI, DAVIN M. VOOT, MARK T. ANDERSEN, IONA E. W. O'BRIEN, AND RICHARD L. S. FORSTER*

Molecular Genetics Group, The Horticultural and Food Research Institute of New Zealand Ltd., Private Bag 92169, Auckland, New Zealand

Communicated by George Bruening, June 23, 1994

ABSTRACT White clover mosaic virus strain O (WCIMV-O), species of the *Potexvirus* genus, contains a set of three partially overlapping genes (the triple gene block) that encodes nonvirion proteins of 26 kDa, 13 kDa, and 7 kDa. These proteins are necessary for cell-to-cell movement in plants but not for replication. The WCIMV-O 13-kDa gene was mutated (to 13*) in a region of the gene that is conserved in all viruses known to possess triple-gene-block proteins. All 10 13* transgenic lines of *Nicotiana benthamiana* designed to express the mutated movement protein were shown to be resistant to systemic infection by WCIMV-O at 1 μg of WCIMV virions per ml, whereas all plants from susceptible control lines became systemically infected. Of the 13* transgenic lines, 3 selected for their abundant seed supply were shown to be resistant to systemic infection when challenged by inoculation with three different WCIMV strains (O, M, and J) or with WCIMV-O RNA at 10 μg/ml. Most plants were also resistant to systemic infection at inoculum concentrations up to 250 μg of WCIMV virions per ml. In addition, the three 13* transgenic plant lines were found to be resistant to systemic infection with two other members of the *Potexvirus* group, potato virus X and narcissus mosaic virus, and the *Carlavirus* potato virus S but not to be resistant to tobacco mosaic virus of the *Tobamovirus* group. These results indicate that virus resistance can be engineered into transgenic plants by expression of dominant negative mutant forms of triple-gene-block movement proteins.

Cell-to-cell and long-distance movement of viruses in plants are not passive events, and most, if not all, plant viruses have a gene or set of genes that mediate movement (1). These movement genes are an essential part of the virus-infection process because plant protoplasts are frequently able to support the replication of viruses that are not able to infect the intact plant (2). Naturally occurring resistance genes may function by interfering with the ability of the invading virus to move from the initially infected cells to surrounding tissues. Although traditional plant breeding has been used successfully to control several virus diseases, natural genes for resistance are not always available to breeders, and single genes for resistance can be overcome by rapidly changing virus populations. As a consequence, genetically engineered protection against viruses in transgenic plants has been adopted as an alternative control strategy. This strategy has been shown to have great potential, but most of the transgenic plants produced to date show resistance against only a limited number of viruses or virus strains (3). Many important crop species, for example potato and rice, are infected by numerous different viruses or virus strains, making the development of superior plant lines resistant to each virus based on these narrow-spectrum resistance factors potentially difficult. New forms of resistance active against several different viruses or groups of viruses are being sought. One such approach involved the introduction of the gene coding for rat 2′-5′ oligoadenylate synthetase into the genome of potato plants (4). Genetically engineering transgenic plants to block virus movement, mimicking the mechanism of some natural resistance genes, has been proposed (1, 5, 6) but remains mostly unexploited.

The movement function of plant viruses can be complemented by another, frequently unrelated, virus in double infections (7). This apparent lack of specificity for the movement function has been demonstrated by De Jong and Ahlquist (8) who showed that the movement protein from a *Tobamovirus* could support the movement requirements of a *Bromovirus*. This nonspecific complementation of the movement process by unrelated viruses suggested to us that inhibition of movement might also be nonspecific, invoking resistance to viruses of different groups. Recently, transgenic tobacco plants [a nonhost of brome mosaic virus (BMV, of the *Bromovirus* group)] expressing the BMV 32-kDa movement protein were found to be resistant to infection by tobacco mosaic virus (TMV, of the *Tobamovirus* group), suggesting that expression of a heterologous movement protein not adapted for a particular host can interfere with the process mediated by the homologous movement protein (5). Transgenic tobacco plants expressing a mutated form of the TMV 30-kDa movement protein were found to be resistant to infection by three strains of TMV (6). However, this resistance was manifest primarily as a delay in infection.

A set of three partially overlapping genes known as the triple gene block encodes nonvirion proteins and is found in the genome of the *Potexvirus*, *Carlavirus*, *Furovirus*, and *Hordeivirus* groups (9, 10) as well as several unclassified plant viruses (11, 12) of the Sindbis virus-like superfamily (13). The triple-gene-block proteins have been shown by site-directed mutagenesis to be essential for virus movement for viruses of the *Potexvirus* (14), *Hordeivirus* (15), and *Furovirus* groups (16). The precise mechanism by which the triple-gene-block proteins mediate the movement process is unclear, however, in all instances the protein encoded by the 5′-most gene of the triple gene block has domains conserved in RNA helicases. All of the proteins encoded by the central gene of the triple gene block have two hydrophobic domains suggestive of transmembrane proteins (9, 10).

The triple gene block of white clover mosaic virus (WCIMV), a member of the *Potexvirus* group, encodes proteins of 26 kDa, 13 kDa, and 7 kDa (10, 17). A WCIMV mutant expressing a mutated 13-kDa gene (13*) was unable to multiply to detectable levels in intact plants but produced wild-type levels of viral RNA species and coat protein when

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: WCIMV, white clover mosaic virus; TMV, tobacco mosaic virus; NMV, narcissus mosaic virus; PVX, potato virus X; PVS, potato virus S.

*To whom reprint requests should be addressed.

inoculated to protoplasts (14), suggesting that the mutations introduced into the 13* gene inactivated the function of the encoded protein. The mutated 13* gene was transferred into the genome of *Nicotiana benthamiana* (a systemic host of WClMV) to determine whether this gene could confer non-specific virus resistance. This paper describes the analysis of transgenic plants expressing the 13* gene. Resistance was observed against systemic infection by three different strains of WClMV, two other *Potexvirus* species, and one *Carlavirus*, but not against a *Tobamovirus*.

MATERIALS AND METHODS

Virus Isolates. WClMV strains O and M (WClMV-O and WClMV-M) were obtained from individual white clover plants from the South Island and North Island of New Zealand, respectively (18). WClMV strain J from Japan was supplied by C. Hiruki (Edmonton, AB). The WClMV strains were maintained in pea, and virions were purified as described (18). Potato virus X (PVX, type species of the *Potexvirus* group) strain NZ1 was obtained from field-infected potato cv. Ilam Hardy, maintained in *Nicotiana tabacum* cv. Xanthi, and virions were purified as described (19). Narcissus mosaic virus (NMV, member of the *Potexvirus* group) was obtained from field-infected narcissus, maintained in *Nicotiana clevelandii* and purified as described for PVX. Concentrations of purified virion preparations were estimated by absorbance at 260 nm (A_{260}). An A_{260} of 3.0 was considered equal to 1 mg of virus per ml. Virions were diluted in 0.25% sodium pyrophosphate buffer (pH 8.0) containing 0.25% Celite and 0.25% bentonite and mechanically inoculated to three carborundum-dusted leaves per plant.

Potato virus S (PVS; a member of the *Carlavirus* group) strain ME was kindly provided by M. Elder (Vancouver), and inoculum was prepared from sap extracted from systemically infected *N. benthamiana* leaves diluted in water at a ratio of 0.02–1 mg of infected tissue per ml of water. By ELISA, 0.02 mg of infected tissue per ml of water corresponded to ≈0.5 μg of virions per ml.

Viral RNA was prepared from purified virions by proteinase K digestion followed by phenol extraction and ethanol precipitation.

DNA Cloning. WClMV-O (17) sequences representing the mutated 13-kDa and wild-type 7-kDa open reading frames (38% of the 7-kDa gene sequence overlaps the 13-kDa gene sequence) were produced by using the oligonucleotides 5'-CCCGGGATCCTGATGCCTTTGATTCCTCC-3' and 5'-CCAAGAATTCGTACGTTCAAATAGATGGCAC-3' and plasmid p13b DNA (14) as template in a PCR reaction. The resulting PCR fragment, WClMV-O nucleotides 4677–5158 (17), was digested with *Bam*HI and *Eco*RI and ligated into the vector pSP72 (Promega) digested with the same two restriction enzymes, producing plasmid p13*-1. This plasmid was digested with *Sal* I and *Cla* I, and the approximately 500-bp fragment containing the WClMV-O 13* gene plus 7-kDa sequences was ligated into plasmid pART7 (20) digested with *Xho* I and *Cla* I, producing plasmid p13*-2. This plasmid was digested with *Not* I and ligated into the *Not* I-digested binary vector pART27 (20), producing plasmid p13*-3. *Agrobacterium tumefaciens* strain LBA4404 was transformed with plasmids pART27 or p13*-3 by the freeze–thaw method (21).

Amino acid sequences were analyzed by using the University of Wisconsin Genetics Computing Group version 5 programs (22).

Transgenic Plants. Leaf discs of *N. benthamiana* were transformed by *Agrobacterium*-mediated transformation by standard techniques (23). Transformed cells were selected on shoot-inducing medium containing 300 μg of kanamycin and 100 μg of cefotaxime per ml. Young shoots were transferred to hormone-free medium containing 100 μg of kanamycin per ml for rooting to occur. Only one shoot per leaf disc was transferred to rooting medium to ensure that independent R_0 generation plant lines were established.

Inheritance of kanamycin resistance was determined by germinating self-pollinated seed (R_1 generation), harvested from regenerated transgenic plants (R_0 generation), on medium containing 300 μg of kanamycin per ml. Approximately 2 week-old kanamycin-resistant R_1 seedlings were transferred to soil and grown under containment glasshouse conditions.

Ten plant lines (13^*_{1-10}) transformed with the mutated WClMV 13* gene plus 7-kDa sequences were established. Control lines, C_{1-3}, were similarly established after transformation with the unmodified binary vector pART27 (20).

Analysis of Transgenic Plants. Northern (RNA blot), Western (immunoblot), and ELISA analyses of uninoculated and inoculated leaves of R_1 plants were performed by standard techniques (24, 25). For Northern blot analysis, ^{32}P-labeled RNA complementary to WClMV-O sequences 3997–5802 (the triple gene block plus coat-protein gene) was used. Western blot analysis involved rabbit anti-coat protein serum produced by injecting New Zealand White rabbits with purified WClMV-O virus particles. For analysis of resistance to viral infection, approximately 6-week-old kanamycin-resistant R_1 plants were challenged by mechanical inoculation either with preparations of virus particles or with virus RNA. Three carborundum-dusted leaves of each plant received 30–50 μl of inoculum per leaf (depending upon leaf size). Plants inoculated with PVX, NMV, or PVS were examined daily for symptom development. Because WClMV does not produce definitive symptoms on *N. benthamiana*, uninoculated leaves from the top of the plant were assayed for the presence of virus by ELISA and/or back-inoculation approximately 12, 30, and 60 days after inoculation to the local lesion assay host *Vigna unguiculata* (cowpea) cv. Blackeye. At least 12 plants per line as indicated (13^*_{1-10} or control C_{1-3} lines) were used per trial, with each trial being repeated at least twice with freshly prepared virus or RNA preparations. Systemic infection of 100% of plants of the control lines C_{1-3} was observed in each trial.

Protoplasts from young leaves of transgenic line 13*$_4$ or control line C_1 were prepared by the procedure of O'Brien and Lindsay (26). WClMV-O RNA (0.1 μg per 10,000 protoplasts) was introduced by electroporation. The replication of WClMV-O in 13*$_4$ and control protoplasts was determined by ELISA analysis of extracts 1 day after inoculation.

RESULTS

Mutation of the WClMV 13-kDa Protein. In all viruses that contain a triple gene block, the second open reading frame of the block encodes a small protein (12–14 kDa) with conserved calculated structural motifs (two hydrophobic domains separated by a hydrophilic domain containing conserved amino acids) (27). The 13-kDa gene of WClMV-O was mutated by using site-directed mutagenesis to encode a 13-kDa protein that contained one phenylalanine and five alanine residues in place of conserved amino acid residues in the central hydrophilic domain (14). These mutations did not disrupt the two hydrophobic domains but were predicted to have an effect on the conserved hydrophilic domain.

Hydrophobicity plots of the WClMV 13-kDa, PVX 12-kDa, NMV 13-kDa, and 13* proteins (Fig. 1) illustrate the conserved nature of their genes. However, the wild-type proteins have a hydrophilic peak at the position of the conserved domain, while the 13* protein has a neutral-to-hydrophobic stretch of amino acids in the same position (indicated by the bar in Fig. 1).

Transgenic Plants. Ten R_0 plants, transformed with the 13* gene construct, were self-pollinated, and R_1 seed was col-

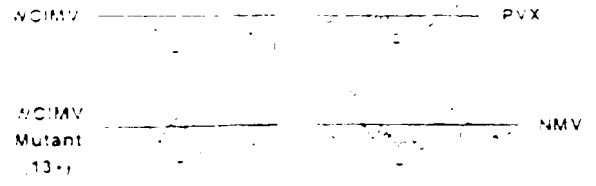


FIG. 1. Hydrophobicity plots of the 13-kDa protein of WCIMV-O, the mutated 13-kDa protein of WCIMV-O (13*), the 12-kDa homologue of PVX, and the 13-kDa homologue of NMV. The bar indicates the location of the conserved amino acid sequence present in the *Potexvirus*, *Carlavirus*, *Furovirus*, *Hordeivirus*, and other virus groups that contain a triple gene block.

lected, establishing plant lines 13^*_{1-10}. Inoculation of plants from all 10 of these lines demonstrated that these plants were resistant to systemic infection by WCIMV-O at an inoculum concentration of 1 μg of virions per ml. In contrast, WCIMV-O was found to infect all of the plants of control lines (C_{1-3}) inoculated with 0.5 μg of virions per ml (the lowest concentration tested). Transgenic plant lines were produced that contained one of nine different transgenes derived from WCIMV-O sequences that encode the wild-type 13-kDa protein plus 7-kDa protein, other wild-type triple-gene-block sequences or gene combinations, or triple-gene-block sequences in an antisense orientation. Of the 49 independent plant lines containing wild-type WCIMV-O sequences that were tested for resistance, all of the plants from 48 lines were susceptible to systemic infection after inoculation with 1 μg of WCIMV per ml (unpublished data). This suggested that the resistant phenotype demonstrated by all 10 13* transgenic lines was due to interference by the 13* mutant protein in a "dominant negative" fashion (28) rather than to the partially overlapping wild-type 7-kDa sequences, to somaclonal variation, or to a RNA-mediated effect. The expression of wild-type movement proteins of other viruses in transgenic plants also has resulted in plants susceptible to infection by the corresponding viruses (29–31).

Based on these results, three 13* transgenic lines that gave good seed supplies were chosen for further analysis (13^*_1, 13^*_2, and 13^*_4). Plants of each of the three lines accumulated similar levels of WCIMV-specific transcript RNA, as determined by Northern blot analysis (Fig. 2). Because of the absence of antisera capable of reacting with the mutant 13*

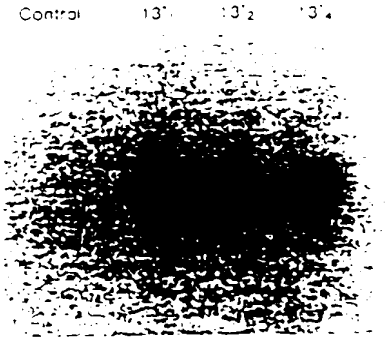


FIG. 2. Northern blot analysis of RNA extracted from leaves of uninoculated plants of the control lines C_1 and C_2 (shown in the first two lanes) and three 13* transgenic lines. The probe was ^{32}P-labeled RNA complementary to the WCIMV triple-gene-block and coat-protein sequences. Unfractionated RNA (10 μg) was loaded per lane.

probe [illegible] to quantitate the [illegible] protein expressed in the 13* transgenic plants. The ratio of green to white seedlings growing on medium containing kanamycin was used to estimate the number of insertion events for the kanamycin-resistance gene present in the 13* transgenic lines. Results suggest that 13^*_1 and 13^*_4 gene expression was from a single locus (3:1 green to white seedlings), and 13^*_2 expression was from two loci (15:1).

Resistance Studies. Plants of the three 13* transgenic lines plus two control lines were challenged with WCIMV-O inocula at 10, 50, or 250 μg of virions per ml. Plants of each of the three 13* transgenic lines were resistant at an inoculum concentration of 10 μg of virions per ml, with none of the plants becoming systemically infected. However, at the higher inoculum levels, some plants eventually became systemically infected, especially in the 13^*_1 line (Fig. 3*A*). Plants of the three 13* transgenic lines were also inoculated with viral RNA to determine whether the resistance was active only against virus particles, as is typical of coat protein-mediated resistance (32). At RNA concentrations of 1 and 10 μg/ml, plants of all three 13* transgenic lines showed resistance against systemic spread of the virus, with none of the 13* plants but all of the control plants becoming systemically infected.

To test whether the 13* transgenic lines were protected against other strains of WCIMV, plants were inoculated with WCIMV strains M and J at concentrations of 10 and 50 μg of virions per ml. WCIMV strain M is 88% homologous to strain O at the nucleotide level (17); strain J has not been sequenced. The response of the 13* transgenic plants to challenge by both strains was similar at all concentrations tested: all 13* plants escaped systemic infection at the lower inoculum concentration (10 μg of virions per ml), while all control plants were systemically infected after postinoculation day 12, with either strains M or J. At the inoculum concentration of 50 μg of virions per ml, plants of the 13* transgenic lines largely escaped systemic infection (Fig. 3*B*), and although some of the 13* transgenic plants eventually became systemi-

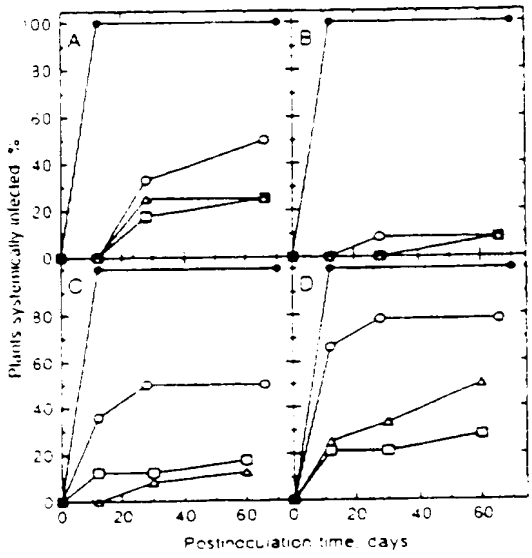


FIG. 3. Time course of development of systemic infection in plants inoculated with 250 μg of WCIMV-O virions per ml (*A*), 50 μg of WCIMV-M virions per ml (*B*), 1 μg of PVX virions per ml (*C*), and 50 μg of PVX virions per ml (*D*). ● Control plants; ○, 13^*_1; △, 13^*_2; □, 13^*_4. Twelve plants per line were used in each experiment.

...infected, there was a significant delay in the development of this infection (Fig. 3B, strain J data not shown).

To determine whether the resistance was also manifest against other members of the *Potexvirus* group, PVX strain NZ1 and NMV strain NZA were also used as challenge inoculum. A significant level of protection was also displayed against both of these *Potexvirus* species at 1 and 10 μg of virions per ml, although the degree of protection was less than that exhibited against the WCIMV strains O, M, or J (Fig. 3 *C* and *D*, NMV data not shown).

In view of the resistant phenotype demonstrated by the 13* transgenic plants against systemic infection with three strains of WCIMV and two other *Potexvirus* species, resistance to challenge by a virus from another group, PVS (a *Carlavirus*), which also contains a triple gene block, was tested. Plants of 13^*_1, 13^*_2, and 13^*_4 lines and control line C_1 were inoculated with diluted extracts from *N. benthamiana* leaves infected with PVS. On plants of the control line, C_1, chlorotic/necrotic local lesions were discernible on the inoculated leaves 4 days after inoculation (>250 local lesions per leaf when using 1 mg of infected tissue per ml of water as inoculum). On inoculated leaves of plants of the 13* transgenic lines, there was a delay until 6 days after inoculation in the development of local lesions. Furthermore, numbers of lesions were significantly reduced (<25 lesions per leaf when using 1 mg of tissue per ml of inoculum) and were approximately half the size of the wild-type local lesions. Systemic symptoms developed on all plants of the control line 6 or 7 days after inoculation for all inoculum concentrations tested, and plants died within 21 days after inoculation; however, systemic symptoms were delayed on plants of the 13* transgenic lines until 8–10 days after inoculation for the stronger inoculum concentrations (0.1–1 mg of infected tissue per ml of water) and failed to develop for the weakest (0.02 mg of infected tissue per ml of water).

To determine whether the resistance manifest against the members of the *Carlavirus* and *Potexvirus* groups was also manifest against viruses that do not possess a triple gene block, control and 13* transgenic lines were also challenged by inoculation with TMV (at 1 μg of virions per ml and 10 μg of virions per ml). No discernible differences in the rate of development of systemic symptoms were observed between the control and 13* transgenic lines.

The step in the infection process inhibited in the 13* transgenic lines could be expected to occur at the level of movement, rather than replication. To test the hypothesis that the inhibition of viral spread in the 13* transgenic plants was due to interference with movement, protoplasts were infected with WCIMV-O RNA by electroporation. ELISA analysis showed a similar level of coat-protein production in the protoplasts from line 13^*_4 and control line C_1 [ELISA A_{405} values for extracts (10,000 infected protoplasts per ml) were 0.32 for 13^*_4, 0.31 for C_1, and 0.005 for mock-inoculated].

Northern and Western blot analyses of leaves inoculated with 10 μg of WCIMV-O RNA per ml also were used to examine this hypothesis. Genomic and subgenomic RNA species indicative of replication were detected by Northern blot analysis in the inoculated leaves of plants from the three 13* transgenic plant lines 7 days after inoculation (Fig. 4) but at a reduced level relative to the control C_1 infection. Although the level of mRNA derived from the 13* transgene was similar in the three 13* transgenic lines (Fig. 2), there was a variable level of genomic RNA produced in the inoculated leaves (Fig. 4). This suggests that replication was not completely blocked in the inoculated leaves, despite the fact that systemic spread did not occur in these plants. WCIMV coat protein was detected by Western blot analysis in the inoculated leaves of 13* transgenic plants, but at a level 1–2 orders of magnitude lower than the amount in the inoculated leaves of C_1 plants (results not shown).

FIG. 4. Northern blot analysis of WCIMV-specific RNA produced in 13* and C_1 leaves inoculated with 10 μg of WCIMV-O RNA per ml. ^{32}P-labeled RNA complementary to the triple-gene-block and coat-protein sequences was used as probe, and 5 μg of unfractionated RNA was loaded per lane. The genomic 6.0-kb RNA and coat protein subgenomic 0.9-kb RNA are indicated. mRNA from the 13* transgene comigrated with the 0.9-kb subgenomic RNA.

DISCUSSION

Ten independent transformed R_1 plant lines, 13^*_{1-10}, designed to express a mutated WCIMV 13-kDa movement protein were established and shown to be resistant to systemic infection by WCIMV-O. Three transgenic 13* lines chosen for further analysis were found to be resistant to systemic infection by two additional strains of WCIMV, two other *Potexvirus* species, PVX and NMV, and a *Carlavirus*, PVS. These viruses all contain the triple gene block and, based on the results with WCIMV (14), are likely to require these genes for movement in plants. Although resistant to four viruses that contain the triple gene block, the 13* transgenic lines were not resistant to infection with TMV. This suggests that the mutant 13* protein has blocked movement by interacting with viral and/or host components required for movement by viruses utilizing the triple-gene-block proteins.

Although the mechanism by which the wild-type WCIMV 13-kDa protein mediates movement is unknown, several possibilities may be proposed. The 13-kDa protein of the *Furovirus* beet necrotic yellow vein virus (33) has been shown to be located in the membranous subcellular fractions. The membranous subcellular fractions of the wild-type 13-kDa transgenic plants were also shown to contain this protein (unpublished data). It has been demonstrated that the 30-kDa movement protein of TMV interacts with plasmodesmatal components, enlarging the molecular exclusion limits (34). The hydrophobic domains and known membrane interaction of the 13-kDa protein suggest that this protein might play a role in modifying the plasmodesmata of host plants. Another possibility is that the 12- to 14-kDa proteins are part of a viral ribonucleoprotein transport complex. The 12- to 14-kDa proteins have a high isoelectric point, indicating that these proteins could have nucleic acid-binding properties. The ability to bind nucleic acids is a characteristic of the movement proteins of TMV (35), cauliflower mosaic virus of the *Caulimovirus* group (36), and red clover necrotic mosaic virus of the *Dianthovirus* group (37). Regardless of the mechanism by which the wild-type 13-kDa protein mediates movement, it is clear that the mutations introduced into the 13* sequence interfere with this ability (14). Furthermore, these mutations are dominant, out-competing the wild-type

[illegible] negative mutant proteins have been previously proposed as a means of generating viral resistance[1–3]. Resistance based on a block of viral movement is a naturally occurring method by which plants mediate viral challenge—e.g. TMV resistance conferred by the *Tm2* gene [38], the N gene-mediated hypersensitive response by tobacco [39], and the broad, general resistance of wild potato to infection by PVX of the *Potexvirus* group and potato virus Y of the *Potyvirus* group [39].

Dominant negative mutations [28] within the viral replicase gene have been reported to provide resistance to viruses [40–42]. These replicase-based dominant mutations have all provided protection exclusively against the homologous or closely related virus strains. Our results indicate that the WCIMV 13° gene is dominant negative and provides non-specific protection to transgenic plants by blocking virus movement.

This example of multiple virus resistance through the expression of a single gene has implications for the development of virus control strategies for crops that are infected by multiple viruses that rely upon the triple-gene-block proteins for movement (such as potato PVX and potato aucuba mosaic virus of the *Potexvirus* group and potato M and potato S viruses of the *Carlavirus* group). Further mutations of the 13-kDa protein may allow a better understanding of the resistance mechanism and the role of the wild-type triple-gene-block proteins in mediating virus movement.

1. Deom, C. M., Lapidot, M. & Beachy, R. N. (1992) *Cell* **69**, 221–224.
2. Daubert, S. (1992) in *Molecular Signals in Plant-Microbe Communications*, ed. Verma, D. P. (CRC, Boca Raton, FL), pp. 423–443.
3. Fitchen, J. H. & Beachy, R. N. (1993) *Annu. Rev. Microbiol.* **47**, 739–763.
4. Taave, E., Aaspollu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teem, T. H., Kelve, M., Seppanen, P. & Saarma, M. (1993) *Bio/Technology* **11**, 1048–1052.
5. Malyshenko, S. I., Kondakova, O. A., Nazarova, J. V., Kaplan, I. B., Taliansky, M. E. & Atabekov, J. G. (1993) *J. Gen. Virol.* **74**, 1149–1156.
6. Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J. & Beachy, R. N. (1993) *Plant J.* **4**, 959–970.
7. Malyshenko, S. I., Kondakova, O. A., Taliansky, M. E. & Atabekov, J. G. (1989) *J. Gen. Virol.* **70**, 2751–2757.
8. De Jong, W. & Ahlquist, P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6808–6812.
9. Morozov, S. Y., Lukasheva, L. I., Chernov, B. K., Skryabin, K. G. & Atabekov, J. G. (1987) *FEBS Lett.* **213**, 438–442.
10. Forster, R. L. S., Bevan, M. W., Harbison, S. A. & Gardner, R. C. (1988) *Nucleic Acids Res.* **16**, 291–303.
11. Kanyuka, K. V., Vishnichenko, V. K., Levay, K. E., Kondenkov, D. Yu., Ryabov, E. V. & Zavriev, S. K. (1992) *J. Gen. Virol.* **73**, 2553–2560.
12. Sumi, S., Tsunevoshi, T. & Furutani, H. (1993) *J. Gen. Virol.* **74**, 1879–1885.
13. [illegible] (1993) *Nucleic Acids Res.* **21**, 4431–4435.
14. Beck, D. L., Guilford, P. J., Voot, D. M., Wright, M. J. & Forster, R. L. S. (1991) *Virology* **183**, 695–702.
15. Petty, I. T. D., French, R., Jones, R. A. & Jackson, A. O. (1990) *EMBO J.* **9**, 3453–3457.
16. Guiner, D., Boucoubaa, S., Herm, A., Gertier, M., Richards, K. & Jonard, G. (1992) *Virology* **189**, 40–47.
17. Beck, D. L., Forster, R. L. S., Bevan, M. W., Bisaro, K. A. & Lowe, S. C. (1990) *Virology* **177**, 152–159.
18. Forster, R. L. S., Guilford, P. J. & Pauler, D. V. (1988) *J. Gen. Virol.* **68**, 181–190.
19. Forster, R. L. S. & Mune, K. S. (1978) *N. Z. J. Agric. Res.* **21**, 237–242.
20. Gleave, A. P. (1992) *Plant Mol. Biol.* **20**, 1203–1207.
21. An, G., Ebert, P. R., Mitra, A. & Ha, S. B. (1988) in *Plant Molecular Biology Manual*, eds. Gelvin, S. B., Schilperoort, R. A. & Verma, D. P. S. (Kluwer, Dordrecht, The Netherlands), pp. 1–19.
22. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
23. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227**, 1229–1231.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
25. Clark, M. F. & Adams, A. N. (1977) *J. Gen. Virol.* **34**, 475–483.
26. O'Brien, I. E. W. & Lindsay, G. C. (1990) *Plant Cell Tissue Organ Cult.* **33**, 31–37.
27. Morozov, S. Y., Dolja, V. V. & Atabekov, J. G. (1990) *J. Mol. Evol.* **29**, 52–62.
28. Herskowitz, I. (1987) *Nature (London)* **329**, 219–222.
29. Deom, C. M., Oliver, M. J. & Beachy, R. N. (1987) *Science* **237**, 389–394.
30. Deom, C. M., Schubert, K., Wolf, S., Holt, C. A., Lucas, W. J. & Beachy, R. N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3284–3288.
31. Deom, C. M., Wolf, S., Holt, C. A., Lucas, W. J. & Beachy, R. N. (1991) *Virology* **180**, 251–256.
32. Beachy, R. N., Loesch-Fries, S. & Tumer, N. E. (1990) *Annu. Rev. Phytopathol.* **28**, 451–474.
33. Niesbach-Klosgen, U., Guilley, H., Jonard, G. & Richards, K. (1990) *Virology* **178**, 52–61.
34. Wolf, S., Deom, C. M., Beachy, R. N. & Lucas, W. J. (1989) *Science* **246**, 377–379.
35. Citovsky, V., Knorr, D., Schuster, G. & Zambryski, P. (1990) *Cell* **60**, 637–647.
36. Citovsky, V. & Zambryski, P. (1991) *BioEssays* **13**, 573–579.
37. Giesman-Cookmeyer, D. & Lommel, S. A. (1993) *Plant Cell* **5**, 973–982.
38. Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H. & Okada, Y. (1989) *Plant Cell* **1**, 515–522.
39. Vaikonen, J. P. T., Penu, E., Jones, M. G. K. & Gibson, R. W. (1991) *J. Gen. Virol.* **72**, 231–236.
40. Longstaff, M., Brignetti, G., Boccard, F., Chapman, S., Baulcombe, D. (1993) *EMBO J.* **12**, 379–386.
41. Anderson, J. M., Palukaitis, P. & Zaitlin, M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8759–8763.
42. Inokuchi, Y. & Hirashima, A. (1987) *J. Virol.* **61**, 3946–3949.

VIROLOGY 189, 40–47 (1992)

XP 002025562

Efficient Cell-to-Cell Movement of Beet Necrotic Yellow Vein Virus Requires 3′ Proximal Genes Located on RNA 2

C. GILMER, S. BOUZOUBAA, A. HEHN, H. GUILLEY, K. RICHARDS, AND G. JONARD[1]

Institut de Biologie Moléculaire des Plantes du CNRS et de l'Université Louis Pasteur, 12 rue du General Zimmer, 67084 Strasbourg Cedex, France

Received January 21, 1992; accepted March [illegible], 1992

RNA 2 of beet necrotic yellow vein virus (BNYVV) carries six open reading frames. The four 3′ proximal frames encode the proteins P42, P13, P15, and P14. The first three species present homologies to proteins encoded by three overlapping open reading frames (the triple gene block) in potexviruses, carlaviruses, and barley stripe mosaic virus. P14 does not display homology with other known plant viral proteins. The functions of P42, P13, P15, and P14 were investigated by site-directed mutagenesis. Full-length transcripts of wild-type BNYVV RNAs 1 and 2 were infectious when coinoculated to protoplasts or leaves of *Chenopodium quinoa*. RNA 2 transcripts in which P42, P13, and P15 were prematurely terminated by frameshift mutations replicated in protoplasts (when inoculated with wild-type RNA 1) but were not infectious to leaves, indicating that the triple gene block proteins of BNYVV are essential for viral cell-to-cell spread. Mutations in P14 were not lethal in leaf infections but smaller local lesions and lesser amounts of viral RNA were produced. RNA 2-related subgenomic RNA species of 2.6, 1.4, and 0.7 kb were detected; they presumably direct synthesis of P42, P13, and P14. No species of the length predicted for a P15-specific subgenomic RNA was detected. © 1992 Academic Press, Inc.

INTRODUCTION

The genome of beet necrotic yellow vein virus (BNYVV) consists of four 3′ polyadenylated positive-sense RNA species referred to in decreasing order of size as RNAs 1 to 4: RNA 1, 6.8 kb; RNA 2, 4.7 kb; RNA 3, 1.8 kb; RNA 4, 1.5 kb (Bouzoubaa *et al.* 1985, 1986, 1987). Some Japanese isolates also contain a fifth component known as RNA 5 (Tamada *et al.* 1989). In the field, BNYVV is transmitted by the soilborne fungus *Polymyxa betae* and is generally found in the roots of sugar beet (Tamada 1975). All four genome components are present in these circumstances (Koenig *et al.* 1986; Lemaire *et al.* 1988; Tamada *et al.* 1989). When mechanically inoculated to leaves of *Chenopodium quinoa* or *Tetragonia expansa*, on the other hand, only RNAs 1 and 2 are required to achieve an infection (Koenig *et al.* 1986; Lemaire *et al.* 1988; Butler *et al.* 1989; Tamada *et al.* 1989). These findings indicate that RNAs 1 and 2 encode basic functions required in all hosts while RNAs 3 and 4 encode products necessary to complete the natural infection process (Tamada and Abe 1989; Tamada *et al.* 1990; Koenig *et al.* 1991).

Each RNA 1 carries a single open reading frame (ORF) with the potential to encode a 207-kDa polypeptide. This gene product is referred to by molecular weight preceded by P. P207 contains helicase

[illegible]

[illegible]

(Gorbalenya *et al.* 1989; Hodgman 1988) and core polymerase (Koonin *et al.* 1989) sequence domains characteristic of viral RNA replicases of the Sindbis-like superfamily (Goldbach and Wellink 1988). RNA 2 carries six ORFs. The 5′ proximal ORF encodes the 21-kDa viral coat protein. The coat protein ORF is in the same reading frame as and separated by a single amber termination codon from a downstream 54-kDa ORF (Fig. 1). Readthrough of the coat protein termination codon into the adjacent ORF occurs about 10% of the time both in vitro and in planta to produce a readthrough protein P75 (Fig. 1) with the coat protein sequence at its N-terminus (Diegel *et al.* 1985; Vaslaton, Diegel *et al.* 1990). P75 has been implicated in virus–vector interactions (Tamada and Abe 1989).

The 3′ proximal portion of RNA 2 encodes polypeptides of 42 kDa, 13 kDa, 15 kDa, and 14 kDa (Fig. 1; P42, the P207 contains a helicase consensus domain near its C-terminus. P13 and P15 possess hydrophobic domains (Morozov *et al.* 1987), while P14 possesses several cysteine-rich regions (Koonin *et al.* 1991) and has been shown to bind zinc ions in vitro (Vaslaton, Hodgen *et al.* 1990). Using serological techniques, P42 and P13 have been detected in a membrane-enriched subcellular fraction of BNYVV-infected leaf tissue, while P14 was present in the cytosolic fraction (Vaslaton, Diegel *et al.* 1990). P15 was not detected in any of these experiments.

P42, P13, and P15 have counterparts in the ge

nomes of potexviruses, carlaviruses, and the hordeivirus barley stripe mosaic virus (BSMV) (Morozov *et al.* 1989). This triad of homologous genes is often referred to as the "triple gene block." The evidence points to a role for the triple gene block proteins of BSMV and white clover mosaic potexvirus (WCIMV) in movement of these viruses from cell to cell in their hosts (Petty *et al.* 1990; Beck *et al.* 1991).

A system for producing infectious *in vitro* transcripts from cDNA clones of each BNYVV RNA is available (Ziegler-Graff *et al.* 1989; Guilley *et al.* 1989). Using these transcripts, we have demonstrated that various mutations in the viral coat protein cistron and in the 54-kDa readthrough domain prevent efficient virion assembly without interfering with local lesion formation on *C. quinoa* and *T. expansa* (Schmitt *et al.* 1992). In this paper we have used mutation-bearing RNA 2 transcripts to study the requirement for the triple gene block proteins and P14 for infection of intact plants and protoplasts. We have also detected three subgenomic RNAs which are presumably important for expression of genes of RNA 2.

MATERIALS AND METHODS

cDNA clones and mutagenesis of BNYVV RNA 2

The recombinant cDNA plasmids pB219 (RNA 1 complete cDNA), pB218 (RNA 2 nucleotides 1–2715; all insert coordinates refer to the complete RNA 2 sequence), pBF14 (RNA 2 nts 2035–4612 plus 3′ poly A tail), and pB35 (RNA 3 complete cDNA) have already been described (Bouzoubaa *et al.* 1986; Ziegler-Graff *et al.* 1988; Guilley *et al.* 1989). The structures of the RNA 2 mutants referred to in this paper are shown in Fig. 1. Mutant F, containing a 935-nt deletion in the 54-kDa ORF, was constructed by cutting pB218 with *Mlu*I and *Bgl*II, filling in the protruding extremities with DNA polymerase I Klenow fragment, and recircularizing the DNA with bacteriophage T4 DNA ligase. A 4-nt insertion in the P42 ORF was created by cutting a unique *Sca*I site (nt 2283) in pB218 with Klenow fragment to produce Mutant H. A 4-nt insertion in the P13 ORF (Mutant I) was fashioned by inserting the sequence ACTC between nts 3449 and 3450 of RNA 2 by oligonucleotide-directed site-specific mutagenesis (Zoller and Smith 1982). Mutagenesis was performed on single-stranded phagemid DNA (Ziegler-Graff *et al.* 1988) of plasmid pB21 (Lemaire *et al.* 1988) which contains insert nts 3335–3993 (Fig. 1) cloned between the *Hin*dIII and *Eco*RI sites of BS(−) (Stratagene). A clone (pB21-I) containing the desired mutation was identified by colony hybridization (Ziegler and Smith 1980) and sequence analysis (Sharp *et al.* 1988). Mutant J was constructed by inserting the *Eco*RI fragment B (Fig. 1) of pBF14 into the *Eco*RI site of pB21-I. A 4-nt insertion in the P15 ORF was constructed by partially digesting pBF14 with *Eco*RI, purifying linear full-length molecules by agarose gel electrophoresis, filling in the protruding extremities with Klenow fragment, and recircularizing with DNA ligase. The desired mutant (Mutant J, Fig. 1) carrying a 4-nt insertion at the *Eco*RI site (nt 3792) of the P15 ORF, was identified by restriction enzyme digestion and sequence analysis.

A 10-nt insertion in the P14 ORF (Mutant L) was created by placing a *Bgl*II linker (pGAAGATCTTC) in the *Sna*BI site (nt 4331). In another construction, the two in-phase initiation codons of P14 were eliminated by site-directed mutagenesis. *Eco*RI fragment B (Fig. 1) from pBF14 was inserted into the *Eco*RI site of BS(−) and mutagenesis was performed on single-stranded phagemid DNA using as mutagen the synthetic oligodeoxyribonucleotide 5′-ATCTACCAGATCTCCATACT-CGTT (complementary to nts 4036–4057; the substitution is underlined and the residues indicated by bold lettering represent an insertion with respect to the wild type sequence). *Eco*RI fragment B carrying the mutations was substituted into pBF14 in place of the wild-type fragment to produce Mutant 2022.

Synthesis and inoculation of transcripts

Full-length transcripts of RNAs 1 and 3 were prepared by bacteriophage T7 RNA polymerase-catalyzed run-off transcription of *Hin*dIII-linearized pB219 and pB35 (Ziegler-Graff *et al.* 1988; Guilley *et al.* 1989). cDNA comprising the complete RNA 2 sequence was constituted by ligating the large *Bam*HI–*Bst*XI fragment of pB218 (or the analogous segments of Mutants F and H, which carries nts 1–2690) to a *Bst*XI–*Sal*I fragment derived from pBF14 (or one of its mutated derivatives) carrying the remainder of the RNA 2 sequence plus the 3′ poly A tail (Guilley *et al.* 1989). The ligation product was then used directly as template for run-off transcription (Guilley *et al.* 1989). The amount of full-length transcript produced was evaluated by agarose gel electrophoresis. Transcripts were inoculated to *C. quinoa* leaves without further purification as described (Guilley *et al.* 1989) but for inoculation to *C. quinoa* protoplasts the DNA template was first eliminated by treatment with DNase (Jupin *et al.* 1992).

Inoculation of protoplasts

Mesophyll protoplasts prepared from young leaves of *C. quinoa* (Jupin *et al.* 1992) were inoculated by co-incubation with 5–10 μg RNA 1 transcript and 2–5 μg RNA 2 transcript per 300,000 protoplasts and main-

tained for 48 hr postinoculation (p.i.) as described (Veidt *et al.*, 1992).

Detection of viral RNAs

Total RNA was isolated from infected protoplasts (Veidt *et al.*, 1992) or leaves (Lemaire *et al.*, 1988). If desired, polyadenylated RNA was purified from the total leaf RNA using a PolyATract mRNA isolation kit (Promega) according to the supplier's instructions. The total plant RNA was analyzed for the presence of viral RNAs by Northern hybridization using ^{32}P-labeled antisense viral RNA probes including the RNA 2-specific probe pB21 as already described (Lemaire *et al.*, 1988). Other RNA 2-specific antisense probes used in this work corresponded to nts 638–2081 (probe ΔBB), nts 2078–2715 (probe 2188g) and nts 3949–4481 (probe ΔAS) (Fig. 5).

Mapping the 5′ termini of subgenomic RNA

Total RNA from virus-infected *T. expansa* leaves was sedimented through a 5–20% sucrose gradient made up in 10 m*M* Tris–Cl, pH 7.5, 1 m*M* EDTA, 0.1% sodium dodecylsulphate. Centrifugation was for 16 hr at 4° in a Beckman SW41 rotor turning at 30 000 rpm. Fractions (0.5 ml) of the gradient were tested for the presence of RNA 2-related subgenomic RNAs by Northern hybridization using ^{32}P-labeled ΔAS as probe. Mapping of the 5′ terminus of RNA 2sub, in the corresponding pooled gradient fractions was by primer extension using a synthetic oligonucleotide primer complementary to nts 4128–4148 essentially as described (Boudonck *et al.*, 1991), except that radioactive label in the run-off cDNAs was provided by [^{35}S]-dATP incorporated into the reaction mix. The sequence ladder was produced by dideoxynucleotide chain termination sequencing (Fichot and Girard, 1990) using reverse transcriptase, gradient-purified full-length RNA 2 as template, and the above-mentioned synthetic oligonucleotide as primer.

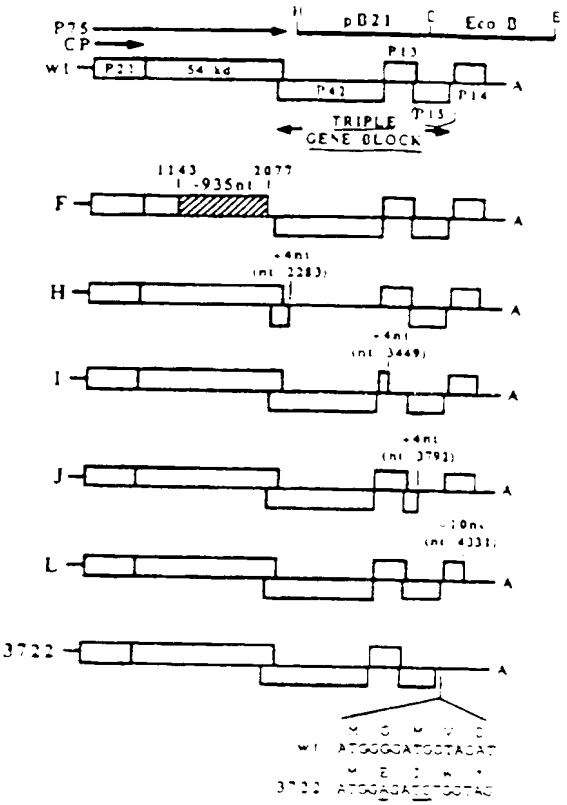


FIG. 1. Structure of the BNYVV RNA 2 mutants used in this study. The structure of wild-type RNA 2 and the position of the ORFs referred to in the text are shown at the top. A represents the 3′ poly(A) tail of the RNA and the heavy arrows indicate the major translation of the coat protein and the P25 readthrough protein. The HindIII–EcoRI insert of pB21 and the EcoRI–EcoRI fragment mentioned in the text are shown to the right of HindIII, EcoRI. The deletion in p3722 is denoted by hatching and the position and nature of the modification in each of the other mutants are indicated. The base substitution in mutant 3722 is underlined and the glycine and the valine codons are underlined with a single line.

RESULTS

Triple gene block mutants are not infectious in plants

Both BNYVV RNAs 1 and 2 are required for infection and production of symptoms (local lesions only on leaves of *C. quinoa* and *T. expansa* (Koenig *et al.*, 1986; Guilley *et al.*, 1989; Tamada *et al.*, 1989). We have already shown that a number of short deletions and other mutations in the 5′ proximal ORFs of RNA 2 (the coat protein cistron and the readthrough domain) do not interfere with infectivity of RNA 2 on leaves (Guilley *et al.*, 1989; Schmitt *et al.*, 1992). In this paper we have tested the effect of mutations (see Fig. 1) in the four proximal ORFs of RNA 2 on infectivity, in repeated experiments. Frameshift mutations designed to disable each member of the triple gene block—P42 (Mutant H), P13 (Mutant I) or P15 (Mutant J; Fig. 1)—produced no symptoms when transcript carrying each mutation was inoculated along with wild-type RNA 1 transcript to leaves of the local lesion hosts *C. quinoa* or *T. expansa*. Furthermore, no viral RNA could be detected in the inoculated leaves by Northern hybridization 5 days post-inoculation (Fig. 2, lanes 3–5). At the same time point, RNA was abundantly present in leaves inoculated with wild-type RNA 1 and 2 transcripts (Fig. 2, lane 2). Some

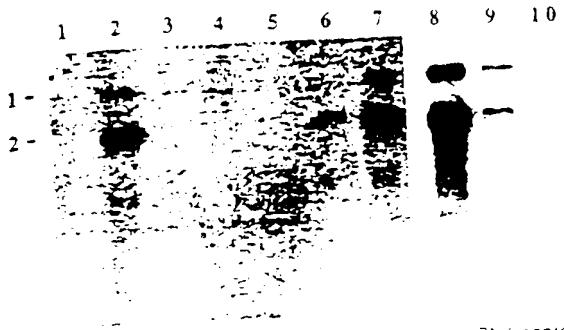


FIG. 2. Analysis by Northern hybridization of the viral RNA contents of *C. quinoa* leaves inoculated with BNYVV RNA 1 transcript and BNYVV RNA 2 transcripts having different structures. The RNA 2 transcript was from Mutant pB218-F (lane 1), wild-type (lanes 2 and 9), Mutant pB218-H (lane 3), Mutant pB218-I (lane 4), Mutant pB218-J (lane 5), Mutant pB218-L (lanes 6, 7, and 8), and Mutant pB218-M (lane 10). In lanes 1–6 the sample corresponded to 200 mg of 3732 (lane 10). In lanes 1–6 the sample corresponded to 200 mg of total leaf RNA. Lane 7 is identical to lane 6 except that 10 times more RNA was loaded. Lanes 8–10 were loaded with material extracted from equal numbers of local lesions. The dry weight of 3732 corresponded to 0.5% of the total RNA extracted. The low yield of RNA 2 relative to RNA 1 in lanes 9–10 was not reproducible. The RNA 1 specific antisense RNA probe was complementary to nts 4740–5880 (Lemaire *et al.* 1988) and the RNA 2 specific antisense RNA probe was obtained from pB2.

lar results were obtained when RNA 3 transcript was included in the inoculum (data not shown).

Other mutations in RNA 2

The P14 ORF was subjected to two different types of mutation. In Mutant L (Fig. 1), a 10-nt insertion was introduced at nt 4331, resulting in premature termination of the ORF by frameshift. A second mutant, 3732, was constructed in which translation of P14 is completely suppressed. In this mutant, a 3-nt insertion and a single-base substitution were introduced at the 5′ terminus of the P14 ORF in such a way as to eliminate one of the two in-phase AUGs at the 5′ terminus and introduce a frameshift after the other (Fig. 1). There are no other in-frame AUGs present in the ORF.

RNA 2 transcripts containing each of the above two mutations were inoculated along with wild-type RNA 1 transcript to *C. quinoa*. Both mutants proved to be infectious, producing local lesions in numbers comparable to those observed in controls with wild-type RNA 2 transcript. The lesions, however, were only about one-third the size of those observed with the wild-type control and consisted of a necrotic center surrounded by a thin chlorotic halo (data not shown). Lesions elicited by wild-type RNA 1 and 2 transcripts have the form of tight circular rings or spots (Bouley *et al.* 1989). Northern hybridization analysis of viral RNA extracted from equal numbers (30) of lesions revealed that the amount of progeny BNYVV RNAs 1 and 2 in leaves infected with the P14 mutants (Fig. 2, lanes 6–7, 9–10) was reduced 10- to 30-fold compared to a control inoculated with wild-type RNA 2 (Fig. 2, lanes 2, 8). Similar results were obtained with an RNA 2 mutant carrying a 96-nt deletion (nts 4287–4384 eliminated) in the P14 ORF (data not shown).

An RNA 2 transcript with a long deletion in the 54-kDa readthrough domain (Mutant F, Fig. 1) was also found to be incapable of infecting leaves (Fig. 2, lane 1). As will be shown below, P42 is probably expressed from a subgenomic RNA. We regard it as likely that the lethal effect of the deletion in Mutant F is due to interference with expression of P42 rather than a requirement for full-length P75 for whole leaf infections (see Discussion).

Infectivity of mutant transcripts in protoplasts

The inability of RNA 2 transcripts carrying mutations in P42, P13, and P15 to infect whole plants may be due to a defect in virus replication, interference with cell-to-cell spread of the virus, or a combination of the two effects. In order to distinguish among these possibilities, protoplast infection experiments were carried out. In preliminary experiments, natural BNYVV RNAs 1 and 2 were introduced into *C. quinoa* protoplasts by electroporation and the time course of appearance of progeny viral RNA was studied by Northern hybridization. After a latent period, newly synthesized viral RNA could be readily detected at 12 hr and continued to accumulate up to 48 hr postinoculation (Fig. 3A). Similar results were obtained when the inoculum was provided as viral RNA transcripts (data not shown). When inoculated alone to protoplasts, the RNA 1 transcript was capable of self-replication (Fig. 3B, lane 1), but the RNA 2 transcript was not (Fig. 3B, lane 2), although it was efficiently amplified when coinoculated with the RNA 1 transcript (Fig. 3B, lane 3). Thus, RNA 2 replication is dependent upon the RNA-dependent RNA polymerase activity supplied by RNA 1.

The RNA 2 transcripts carrying the frameshift mutations in the triple gene block proteins, P42 (Mutant H), P13 (Mutant L), and P15 (Mutant J), and in P14 (Mutants L and 3732) all replicated in protoplasts when coinoculated with transcript of RNA 1 (Fig. 4, lanes 2–6), as did the transcript carrying the large deletion in the readthrough domain (Mutant F, Fig. 4, lane 1). We conclude that the failure of the triple gene block mutants to multiply in whole leaves is not due to destruction of cis-acting signals involved in RNA 2 replication, as can be seen in Fig. 4. However, that several of the mutants tested, most notably the P14 mutant 3732 (Fig. 4, lane

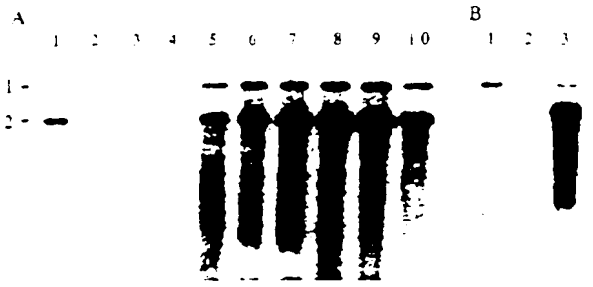


FIG. 3. Infection of *C. quinoa* protoplasts with BNYVV RNA analyzed by Northern hybridization. (A) Time course of appearance of progeny viral RNA after infection. Ten nanograms of purified BNYVV viral RNA (lane 1). RNA extracted from mock-inoculated protoplasts (lane 2) or protoplasts inoculated by electroporation with viral RNA isolate Stras 12 (Quillet et al., 1989) and harvested at different times postinoculation: 1 hr p.i. (lane 3); 6 hr p.i. (lane 4); 12 hr p.i. (lane 5); 24 hr p.i. (lane 6); 36 hr p.i. (lane 7); 48 hr p.i. (lane 8); 72 hr p.i. (lane 9); and 96 hr p.i. (lane 10). The RNA loaded in each sample corresponded to the contents of about 50,000 protoplasts. (B) Protoplast RNA extracted 48 hr p.i. after electroporation with RNA 1 transcript (lane 1), transcript of wild-type RNA 2 (lane 2), and a mixture of the two transcripts (lane 3). Probes were as in Fig. 2.

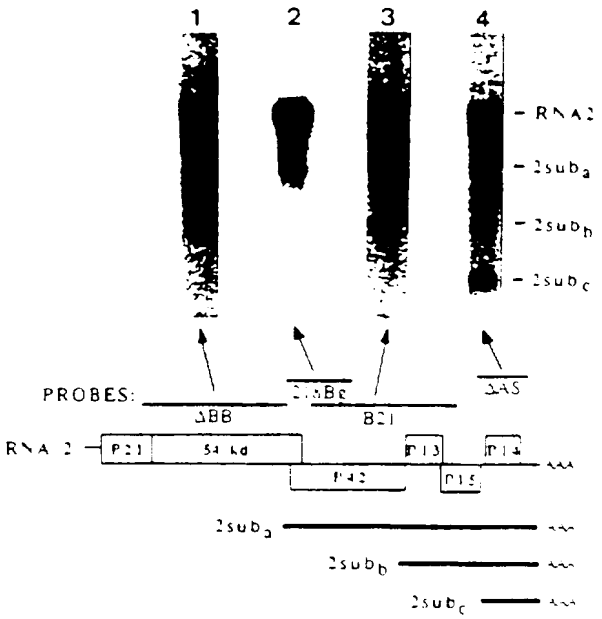


FIG. 5. Detection of BNYVV RNA 2-related subgenomic RNAs by Northern hybridization. Weight-balanced series of a dense and a pale leaf were dated with petiole samples (~200 mg) of Tetragonia tetragonioides leaves infected with BNYVV isolate Stras 12. After transfer of nucleic acids to the membrane, the immobilized RNA was hybridized with DNA strand-antisense RNA probes corresponding to different regions of RNA 2 as indicated in the diagram at the figure (see also "Materials and Methods").

5), aborted abruptly altered the amounts and proportions of RNA 1 and 2 accumulating in the infected protoplasts. The significance of this observation is the subject of ongoing investigations.

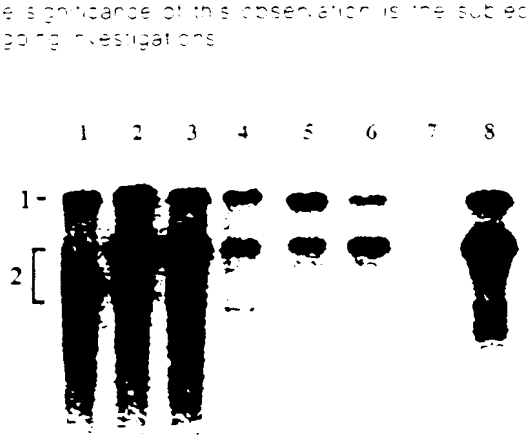


FIG. 4. Time course Northern hybridization of different RNA components of *C. quinoa* protoplasts electroporated with BNYVV RNA 1 together with BNYVV RNA 2 transcripts having different structures. The cells were harvested 48 hr p.i. The RNA 2 transcript was from mutant pBB (lane 1), mutant pBB/B (lane 2), mutant pBB/B (lane 3), mutant pBB/B (lane 4), mutant pBB/B (lane 5), mutant pBB/B (lane 6), and wild type (lane 7). Lanes 8 and 9 contained RNA extracted from mock-inoculated protoplasts. For details, see Fig. 3.

Subgenomic RNAs derived from RNA 2

In plus-strand RNA viruses, 3′ proximal ORFs are generally expressed from subgenomic RNAs which are 3′ coterminal with the genomic RNA. In order to detect such species for BNYVV RNA 2, total RNA was extracted from virus-infected leaves of *T. expansa* and RNA 2-related putative subgenomic RNAs were detected by Northern hybridization using ^{32}P-labeled RNA probes complementary to different parts of the RNA 2 molecule. A probe restricting the 3′ terminal portion of RNA 2 (probe ΔAS, complementary to nts 3949–4428) revealed the existence of three smaller species of approximate sizes 2.6 kb (2sub$_a$), 1.4 kb (2sub$_b$), and 0.7 kb (2sub$_c$) (Fig. 5, lane 4) in addition to the genomic RNA 2. The same three species were also present in RNA extracted from BNYVV-infected *C. quinoa* leaves (data not shown), but were less abundant than in *T. expansa* extracts. Consequently, the latter host was used as the RNA source in further experiments. The small RNA 2-related RNAs could specifically be immunoprecipitated by antibodies that they possess (data not shown).

(data not shown). Probe pB21 (complementary to nts 2335–3793) hybridized with $2sub_a$ and $2sub_c$ but not with $2sub_b$ (Fig. 4, lane 3), while probe 2'88g (complementary to nts 2078–2715) hybridized only with $2sub_a$ (Fig. 5, lane 2). Probe ΔBB, which is complementary to nts 638–2081, did not detectably hybridize with any of the aforesaid bands (Fig. 5, lane 1). Along with $2sub_a$, $2sub_b$, and $2sub_c$, several minor bands were also detected on the Northern blots (Fig. 5, lane 4). Some of these bands, at least, are probably due to ribosomal RNA shadowing (Palukaitis *et al.*, 1983) but we cannot strictly rule out the existence of additional, minor RNA species.

From their estimated lengths, the 5′ termini of $2sub_a$, $2sub_b$, and $2sub_c$ are predicted to map near the beginning of the 42-kDa, 13-kDa, and 14-kDa ORFs, respectively. The 5′ terminus of the most abundant of the putative subgenomic RNAs, $2sub_c$, was mapped with more precision by primer extension. Total RNA from infected leaves was centrifuged through a sucrose gradient and fractions enriched in $2sub_c$ were pooled. The RNA was then hybridized with an oligodeoxyribonucleotide primer complementary to the RNA 2 sequence about 100 nt downstream of the subgenomic RNA's predicted 5′ end and the primer was extended with reverse transcriptase. The length of the resulting run-off cDNA was estimated by comparing its electrophoretic mobility to a sequence ladder in a polyacrylamide sequencing gel. Two major run-off bands differing by 1 nt in mobility were present (Fig. 6). The position of the more rapidly migrating species corresponds to a 5′ terminus for $2sub_c$ at nt 3992, 51 nt upstream of the beginning of the 14-kDa ORF. The slower run-off band may correspond to an additional nucleotide incorporated in response to a 5′ terminal cap structure (Ahlquist and Janda, 1984).

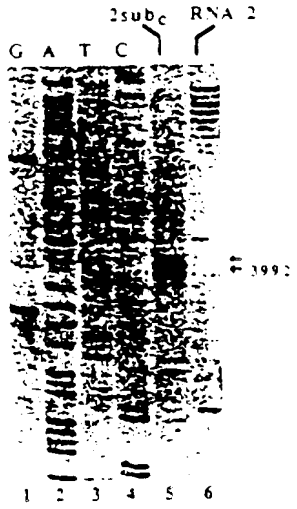


FIG. 6. Mapping the 5′ terminus of $2sub_c$ by primer extension using reverse transcriptase. Lane 5 shows the reverse transcriptase generated run-off transcripts obtained when gradient purified $2sub_c$ was used as template and an oligonucleotide complementary to nts 4128–4148 of BNYVV RNA 2 was the primer. Lane 6 shows a similar reaction but with full-length RNA 2 as template. Lanes 1–4 contain a sequence ladder generated by the dideoxynucleotide chain-termination technique. The pair of abundant run-off products in lane 5 which correspond to the 5′ terminus of $2sub_c$ (at nt 3992) are indicated by arrows.

DISCUSSION

In this paper we have shown that BNYVV RNA 2 transcripts carrying frameshift mutations in each of the triple gene block proteins are amplified in protoplasts when inoculated with BNYVV RNA 1. It follows that none of the aforesaid mutations inactivate cis-acting replication signals on RNA 2 and that none of the triple gene block proteins are required for viral RNA replication. The failure of these same mutants to productively infect whole leaves is thus strong circumstantial evidence that the BNYVV triple gene block proteins are involved in cell-to-cell spread of the infection, a process that is known to require the intervention of virus-coded proteins in a number of cases (Hull, 1989). As noted in the Introduction, a similar conclusion has been drawn for the triple gene block proteins of BSMV and WCMV (Petty *et al.*, 1990; Beck *et al.*, 1991).

The deletion mutant in the readthrough domain, Mutant F, resembles the triple gene block mutants in that it replicated in protoplasts when coinoculated with wild-type RNA 1 transcript but did not support a whole-leaf infection. This deletion (nt 1143–2207 deleted) is predicted to produce a truncated readthrough protein that diverges from the wild-type amino acid sequence after amino acid residue 333 (numbering starts with the coat protein N terminus) and terminates shortly thereafter (following a short missense sequence due to the frameshift). One possible explanation for the failure of Mutant F to replicate in leaves is that P75 or at least its C-terminal portion is also required for cell-to-cell movement. However, observations with another mutant carrying a deletion in the readthrough domain (Schmitt *et al.*, 1992) argue strongly against this hypothesis. The mutant in question, pB21S-ΔM1, carries a short deletion near the 5′ end of the readthrough domain (nts 1065–1163 deleted). Due to frameshift, the P75 sequence in pB21S-ΔM1 diverges from that of the wild-type at amino acid residue 333 and terminates shortly thereafter. This mutant was infectious on leaves (Schmitt *et al.*, 1992), indicating that translation

of the portion of P75 downstream of residue 333 is not required for cell-to-cell movement. Thus the data suggest that the large deletion in Mutant F is lethal in leaf infections because it has eliminated cis-acting RNA sequences. In particular, the 3′ boundary of the deletion in Mutant F maps only 55 nts upstream of the beginning of the P42 ORF and it is likely that the mutation is lethal in leaves because it eliminates the promoter sequence required for transcription of $2sub_b$ and subsequent expression of P42.

Among other viruses containing a triple gene block, there is abundant evidence for a subgenomic RNA encoding the first gene of the triad (Guilford and Forster 1986; Dolja *et al.* 1987; Bendena *et al.* 1987; Mackie *et al.* 1988; Petty *et al.* 1990; Beck *et al.* 1991) but the mode of expression of the second and third genes is not well understood. In the case of BNYVV, candidate subgenomic RNAs have been detected for expression of the first two ORFs of the triple gene block. We have not, however, been able to detect a subgenomic RNA of appropriate size to direct synthesis of P15, raising the possibility that the third ORF is expressed from $2sub_b$ by an unconventional mechanism. Possible mechanisms could include leaky initiation of translation at the upstream (P13) ORF by scanning ribosomes (Mordoc *et al.* 1991), reinitiation on the P15 ORF after termination of P13, or translational frameshifting in the region of overlap between the P13 and P15 ORFs. P14, on the other hand, is apparently expressed from a distinct, relatively abundant subgenomic RNA.

The results presented in this paper reveal that P14 is not required for infection of leaves although in its absence lesions were markedly reduced in size and had necrotic centers. The cause of the tissue necrosis provoked by the P14 mutants is unknown but it cannot be due to a cytotoxic effect of accumulation of the C-terminally truncated form of P14 predicted for Mutant E since the same phenotype is produced by Mutant D120 in which translation of P14-related polypeptides should be completely suppressed. Possibly, the altered lesion phenotype is a consequence of deregulation of expression of other BNYVV gene products in the absence of functional P14. See below. Leaves infected with the P14 mutants accumulated 5- to 30-fold less viral RNA than leaves infected with wild-type RNA 2. The smaller lesion size and the necrotic phenotype characteristic of the P14 mutants may account in part for, but surely not for the lower viral RNA content. It should be recalled, however, that mutations in P14 also significantly diminish accumulation of viral RNAs in protoplast infection experiments (see Figure 4).

The decrease in lesion size in the absence of functional P14 presumably reflects its effect on cell-to-cell movement although the inhibition is not total as in the case of the mutations in the triple gene block. The mechanism by which P14 might intervene in movement is not yet known but in many respects our findings with P14 resemble the observations of Petty *et al.* (1990) concerning the 17-kDa γb protein of BSMV. The γb protein is dispensable for BSMV infection of plants but, in its absence, viral movement is impaired and intracellular accumulation of viral RNA and major viral-coded proteins, including coat protein, is markedly reduced. It has been suggested (Petty *et al.* 1990) that γb *trans*-activates expression of other BSMV proteins and a similar phenomenon may account for our observations with the BNYVV P14 protein. For example, in the absence of functional P14 the BNYVV triple gene block proteins may be expressed at low levels in a constitutive fashion but wild-type P14 positively regulates their expression, either at the level of subgenomic RNA synthesis or translation. Further experimentation will be aimed at testing this hypothesis.

ACKNOWLEDGMENTS

The authors thank Danièle Scheidecker and Jacqueline Marbach for their technical assistance. This work was partially carried out in the context of EEC BRIDGE Program BIOT-CT90-0160.

REFERENCES

Ahlquist, P., and Kaesberg, P. (1984). cDNA cloning and infectious transcription of the complete brome mosaic virus genome. *Mol. Cell. Biol.* 4, 2876–2882.

Beck, D. L., Guilford, P. J., Voot, D. M., Andersen, M. T., and Forster, R. L. S. (1991). Triple gene block proteins of white clover mosaic potexvirus are required for transport. *Virology* 183, 695–702.

Bendena, W. G., Bancroft, J. B., and Mackie, G. A. (1987). Molecular cloning of the lily, yellow mosaic virus RNA: identification of the coat protein coding sequences. *Virology* 157, 377–384.

Boudeau, S., Guilley, H., Richards, K., and Putz, C. (1988). Nucleotide sequence analysis of RNA 1 and 2 of beet necrotic yellow vein virus isolates P2 and G1. *Gene* 68, 1553–1564.

Boudeau, S., Wiesbach-Godden, C., Turpin, C., Guilley, H., Richards, K., and Jonard, G. (1991). Sharpened primary structure of beet necrotic yellow vein virus RNA 3 and 4. *Gene* 102, 269–273.

Boudeau, S., Guilley, H., Guilley, E., Jonard, G., and Richards, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA 1. *Gene* 58, 615–626.

Boudeau, S., Declerc, J., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1988). Nucleotide sequence of beet necrotic yellow vein virus RNA 2. *Gene* 67, 269–280.

Dolja, V. V., Dranca, D. F., Nikovics, D. V., and Atabekov, J. G. (1987). Potato virus X: a single-and double-gene-block virus that encodes a putative nucleoside triphosphate-binding protein required for cell-to-cell transport. *FEBS Lett.* 214, 308–312.

Richert, C., and Braam, W. (1990). An improved method to determine the sequence of RNA using gel-purified primer. *Biotechniques* 8, 50–52.

GOLDBACH, R., and WELLINK, J. (1988). Evolution of plus strand RNA viruses. *Intervirology* 29, 260–267.

GORBALENYA, A. E., KOONIN, E. V., DONCHENKO, A. P., and BLINOV, V. M. (1988). A conserved NTP-motif in putative helicases. *Nature* 333, 22.

GUILFORD, P. J., and FORSTER, R. L. S. (1986). Detection of polyadenylated subgenomic RNAs in leaves infected with the potexvirus daphne virus X. *J. Gen. Virol.* 67, 83–90.

HODGMAN, T. C. (1988). A new superfamily of replicative proteins. *Nature* 333, 22–23.

HULL, R. (1989). The movement of viruses in plants. *Annu. Rev. Phytopath.* 27, 213–240.

KOENIG, R., BURGERMEISTER, W., WEICH, H., SEBALD, W., and KOTHE, C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in sugar beet roots but not in leaves from several plant species. *J. Gen. Virol.* 67, 2043–2046.

KOENIG, R., JARAUSCH, W., LI, Y., COMMANDEUR, U., BURGERMEISTER, W., GEHRKE, M., and LUDDECKE, P. (1991). Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanically inoculated sugarbeets. *J. Gen. Virol.* 72, 2243–2246.

KOONIN, E. V., BOYKO, V. P., and DOLJA, V. V. (1991). Small cysteine rich proteins of different groups of plant RNA viruses are related to different families of nucleic acid binding proteins. *Virology* 181, 395–396.

LEMAIRE, C., MERDINOGLU, D., VALENTIN, P., PUTZ, C., ZIEGLER-GRAFF, V., GUILLEY, H., JONARD, G., and RICHARDS, K. (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa betae*. *Virology* 162, 232–235.

MACKIE, G. A., JOHNSTON, R., and BANCROFT, J. B. (1988). Single and double stranded viral RNAs in plants infected with the potexviruses cactus mosaic virus and foxtail mosaic virus. *Intervirology* 29, 170–177.

MOROZOV, S. Y., DOLJA, V. V., and ATABEKOV, J. G. (1989). Probable reassortment of genomic elements among a cloned RNA containing plant viruses. *Mol. Evol.* 29, 50–62.

MOROZOV, S. Y., LUKASHEVA, L. I., CHERNOV, B. K., SKRYABIN, K. G., and ATABEKOV, J. G. (1987). Nucleotide sequence of the open reading frame adjacent to the coat protein cistron in potato virus X genome. *FEBS Lett.* 213, 438–442.

MOROZOV, S. Y., MIROSHNICHENKO, N. A., SOLDATEV, A. S., FEDORKIN, O. N., ZELENINA, O. A., LUKASHEVA, L. I., KARASEV, A. V., DOLJA, V. V., and ATABEKOV, J. G. (1990). Expression strategy of the potato virus X triple gene block. *J. Gen. Virol.* 72, 2039–2042.

NIESBACH-KLOSGEN, U., GUILLEY, H., JONARD, G., and RICHARDS, K. (1990). Immunodetection of virus-induced beet necrotic yellow vein virus encoded proteins. *Virology* 178, 50–61.

PETTY, I. T. D., FRENCH, R., JONES, P. W., and JACKSON, A. O. (1990). Deletion of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. *EMBO J.* 9, 3453–3457.

PALUKAITIS, P., GARCIA-ARENAL, F., SULZINSKI, M. A., and ZAITLIN, M. (1983). Replication of tobacco mosaic virus VII. Further characterization of single- and double-stranded virus-related RNAs from TMV-infected plants. *Virology* 131, 533–545.

POCH, O., SAUVAGET, I., DELARUE, M., and TORDO, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* 8, 3867–3874.

QUILLET, L., GUILLEY, H., JONARD, G., and RICHARDS, K. (1989). *In vitro* synthesis of biologically active beet necrotic yellow vein virus RNA. *Virology* 172, 293–301.

SCHMITT, C., BALMORI, E., JONARD, G., RICHARDS, K. E., and GUILLEY, H. (1992). *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75 K-dalton readthrough protein is important for efficient viral assembly. *Proc. Natl. Acad. Sci. USA*, in press.

TAMADA, T. (1975). Beet necrotic yellow vein virus. CMI/AAB Description of Plant Viruses No. 144.

TAMADA, T., and ABE, H. (1989). Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* 70, 3391–3398.

TAMADA, T., and KUSUME, T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* 72, 1497–1504.

TAMADA, T., SATO, M., KIGUCHI, T., and KUSUME, T. (1990). Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. In: Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors (R. Koenig, Ed.), pp. 41–44. Eugen Ulmer GmbH, Stuttgart.

TAMADA, T., SHIRAKO, Y., ABE, H., SAITO, M., KUSUME, T., and HARADA, T. (1989). Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. *J. Gen. Virol.* 70, 3399–3409.

VEIDT, I., BOUZOUBAA, S. E., LESER, R. M., ZIEGLER-GRAFF, V., GUILLEY, H., RICHARDS, K., and JONARD, G. (1992). Synthesis of full-length transcripts of beet necrotic yellow vein virus RNA: Messenger properties and biological activity in protoplasts. *Virology* 186, 193–200.

WANG, H., SCHOLL, P., BROWSE, J., and SOMERVILLE, C. (1988). Double stranded DNA sequencing as a choice for DNA sequencing. *Nucleic Acids Res.* 16, 1220.

ZIEGLER-GRAFF, V., BOUZOUBAA, S., GUILPIN, I., GUILLEY, H., JONARD, G., and RICHARDS, K. (1988). Biologically active transcripts of beet necrotic yellow vein virus RNA-3 and RNA-4. *J. Gen. Virol.* 69, 2351–2357.

ZIEGLER, V., RICHARDS, K., GUILLEY, H., JONARD, G., and PUTZ, C. (1985). Cell-free translation of beet necrotic yellow vein virus readthrough of the coat protein cistron. *J. Gen. Virol.* 66, 2079–2087.

ZOLLER, M., and SMITH, M. (1982). Oligonucleotide directed mutagenesis using M13-derived vectors: An efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Res.* 10, 6487–6500.

Plant Cell Reports (1995) 15:91 -96

Plant Cell Reports
Springer-Verlag 1995

Genetically engineered resistance to potato virus X in four commercial potato cultivars

Huimin Xu [1], Houri Khalilian [1], Mohamed Eweida [1], Sam Squire [2], and Mounir G. Abouhaidar [1]

[1] Department of Botany, University of Toronto, 25 Willcocks St., Toronto, Ontario, M5S 3B2, Canada
[2] Ontario Ministry of Agriculture, Food and Rural Affairs, 449 Dunlop St., Barrie, Ontario, L4N 1C3, Canada

Received 17 January 1995/Revised version received 27 March 1995 – Communicated by W. A. Parrott

Abstract. The genes for the capsid protein (CP) and the 8K movement protein of PVX were introduced into potato (*Solanum tuberosum* L.) and expressed under the control of CaMV 35S promoter using a binary vector and *Agrobacterium tumefaciens*. Four commercial potato cultivars (Russet Burbank, Shepody, Desirée and Bintje) have been efficiently transformed. Eleven independent transgenic clones, with CP expression levels higher than 0.05% of the soluble leaf proteins, were analyzed for resistance to inoculation with PVX (5 and 50 μg/ml). The resistance of the transgenic plants to PVX was observed with the lower titer of virus inoculation (5 μg/ml) but not with higher titer (50 μg/ml). A significant reduction in the accumulation of virus in the inoculated transgenic potato plants has been observed under greenhouse and field conditions. Furthermore, the CP gene is very stable and is transferred to new plants originated from stem cuttings or from tubers. The transgenic plants appeared to be phenotypically identical to the nontransformed controls.

Abbreviations: BAP = benzyl-aminopurine, BCIP = 5-bromo-4-chloro-3-indolylphosphate p-Toluidine salt, CaMV = cauliflower mosaic virus, CP = capsid protein, GA_3 = gibberellic acid, Kbp = kilobase pair, NAA = naphthalene acetic acid, NBT = nitroblue tetrazolium chloride, NOS = nopaline synthase, NPT II = neomycin phosphotransferase II, PMSF = phenyl methyl sulfonyl fluoride, PVX = potato virus X, PVY = potato virus Y

Introduction

Potato virus X (PVX) is the most widespread of the potato viruses and often completely infects certain commercial stocks, causing yield reductions estimated to more than 15% (Torrance *et al.* 1986). PVX may be latent, with foliage symptoms or effect on plant vigor detectable only when closely compared to PVX-free stocks. Co-infections of plants with PVX and PVY may show a synergistic increase in disease symptoms and severity (Vance 1991). PVX is, with few exceptions, transmitted vegetatively by tubers in susceptible cultivars. Transmission through sap inoculation is accomplished by contact of plant parts in the field due to wind, animals or machinery.

In plant cross-protection tests, strains of PVX may protect against the effects of one another completely, partially or not at all (Matthews, 1949). The application of cross-protection in controlling plant virus diseases has been practiced in some countries and has met with some success. The classical cross-protection, however, is not recommended as a general practice because the so-called mild strains often reduce yield by about 5 - 10%, the dominant strain of a virus may change to a more virulent strain, and the dangers of double infections still exists (Fulton 1986). However, cross-protection induced by genetic engineering can greatly overcome these problems associated with classical cross-protection. Plants genetically engineered to express the coat protein gene or other nonstructural gene of a plant virus can be protected against infection by the same or related viruses (Fitchen and Beachy 1993; Donson *et al.* 1993; Fang and Grumet 1993; Nakajima *et al.* 1993; Farinelli and Malnoë 1993; Rubino *et al.* 1993; Audy *et al.* 1994).

Potato plants genetically engineered to express the coat protein genes of PVX (Hemenway *et al.* 1988; Hoekema *et al.* 1991; Lawson *et al.* 1990; Jongedijk *et al.* 1992) can also be protected against infections by this virus. Unlike some other forms of transgene-mediated resistance (Fitchen and Beachy 1993), CP-mediated resistance to PVX is dependent on the amount of coat protein made in the transgenic plants, and the mechanisms of protection may be similar to that of classical cross-protection. The viral coat protein

BNSDOCID: <XP 617900A > *ndence to:* M. G. Abouhaidar

encoded in the transgenic plants might prevent the challenging virus's disassembly, recoat the viral RNA as it is stripped, interfere with the putative receptor site on the host, and/or act by some other as yet unidentified mechanisms (Hemenway *et al.* 1988).

In this study the 8K protein and CP genes of PVX were cloned between the CaMV 35S promoter and the NOS transcription terminator. These two genes were then placed into a binary vector and used to transform four potato cultivars, Russet Burbank, Desirée, Shepody and Bintje. The resulting transgenic potato plants were analyzed for the expression of viral coat protein and for their resistance to PVX infection both in the greenhouse and in the field.

Materials and methods

Construction of recombinant plasmid with a subclone of PVX cDNA. A 1.3 Kbp fragment of PVX cDNA (clone 117A, Eweida *et al.* 1990) containing the sequences for the 8K protein gene, the CP gene and the 3' end non-coding region was introduced into the pBIN19 vector, Polyrok I (Bevan 1984) between the CaMV 35S promoter and the NOS terminator (Fig.1), amplified in *Escherichia coli* JM 101 and transferred into and amplified in *E. coli* HB 101. The T-DNA in Polyrok I contains NPT II gene, which is driven by the NOS promoter, in addition to a multiple cloning site bordered by the CaMV 35S promoter and a NOS polyadenylation site (terminator). Conjugation of *A. tumefaciens* strain LBA 4404 and *E. coli* was performed in a tri-parental mating process. The donor strain *E. coli* HB101 harbouring the recombinant plasmid Polyrok I, containing the PVX coat protein gene and the helper strain, *E. coli* DH5α (pBRK 4013), were used in the triple mating process. Transformants were selected on LB plates containing kanamycin (150 µg/ml), nalidixic acid (150 µg/ml) and streptomycin (375 µg/ml). For plant transformation, *A. tumefaciens* (LBA 4404) containing recombinant Polyrok I with PVX coat-protein gene was grown on selective plates at 25 - 28°C. Single colonies were grown overnight in liquid LB medium with 100 µg/ml kanamycin to 10^8 cells/ml.

Transformation/Regeneration from potato tubers. Virus-free potato tubers of Desirée, Russet Burbank, Shepody and Bintje were stored in the dark at 4°C. Potato tubers were washed, peeled and surface sterilized for 20 min in a 1% sodium hypochlorite solution. Tubers were then rinsed thoroughly in large volumes of sterile water. Discs 1-2 mm thick were sliced from cylinders of tuber tissue prepared with a cork borer (10 mm in diameter). Approximately 5 µl of agrobacteria were applied to the surface of potato discs placed on the surface of filter papers (Whatman 1 MM) laid on MS1 medium containing MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg *et al.*, 1968) with 1.5% sucrose at pH 5.7 and 0.9% Difco Bactoagar in a 12 × 90 mm petri dish. After 48 hr of co-culture at 28°C in the darkness, potato discs were transferred onto the selection medium (MS31) containing MS salts, B5 vitamins, 3% sucrose, 1 µg/ml kinetin, 1 µg/ml BAP, 0.5 µg/ml NAA, 100 µg/ml kanamycin, 400 µg/ml carbenicillin and 0.9% agar. Primary shoots that developed after 3 - 4 weeks were cut off, and the remaining discs were cultured again in the same medium. Secondary shoots obtained after 2-3 subcultures were transferred to MS8 medium containing MS salts, B5 vitamins, 3% sucrose, 0.5 µg/ml GA_3, 0.5 µg/ml kinetin, 100 µg/ml kanamycin, 400 µg/ml carbenicillin and 0.9% agar. Rooted plants were transferred to soil and kept under polyethylene bags and high humidity for about two weeks before transferring to greenhouse conditions.

Transformation/Regeneration from potato stems. Axenic cultures of potato cultivars were propagated *in vitro* through shoot-tip cuttings and maintained in MS8 medium. Internodal stem explants were cut 0.5 cm in length, then cut longitudinally and transferred to the bacterial suspension for 2 min, blotted on the surface of sterile filter papers (1 MM) and transferred to petri dishes containing MS1 medium. Explants (10 per plate) were co-cultivated with *A. tumefaciens* for 48 hr. Explants were then washed in MS medium containing 1 g/L carbenicillin for 24 hr, before being blotted dry and cultured in MS7 medium containing MS salts, B5 vitamins, 1% sucrose, 1% glucose, 1% mannitol, 1 µg/ml BAP, 0.5 µg/ml zeatin, 0.5 µg/ml NAA, 400 µg/ml carbenicillin, 100 µg/ml kanamycin and 0.9% Bactoagar. $AgNO_3$ (10 µg/ml) was included in MS7 medium for Russet Burbank (De Block 1988). Regenerated shoots were rooted and transferred to soil as described above. All potato cultures were grown in a culture room at 22/15°C (day/night), with a 16-hr photoperiod at 43 μEm^{-2} sec^{-1} light intensity (white fluorescent).

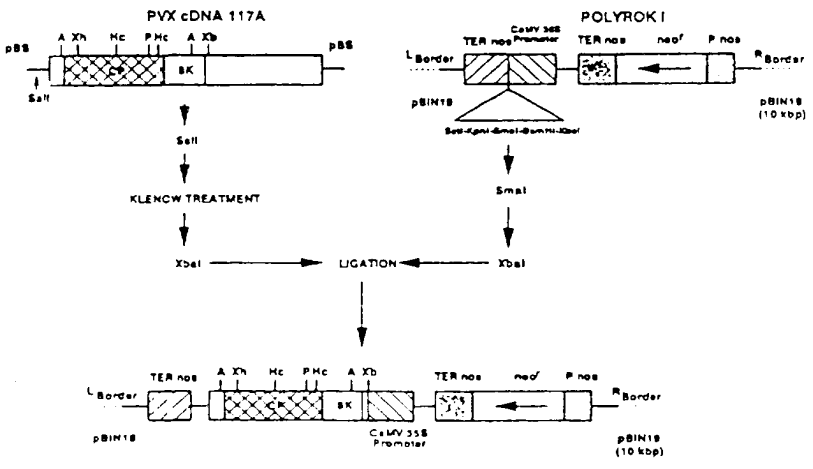


Fig.1. Schematic representation of the arrangement of PVX CP cistron in the vector Polyrok I. Plasmid was digested with *Sma*I and *Xba*I between the CaMV 35S promoter and NOS terminator. PVX cDNA clone 117A in pBS⁺ was first digested with *Sal*I and then treated with DNA polymerase I (Klenow fragment) to render this end blunt. Subsequently the DNA was digested with *Xba*I and ligated prior to transformation in *Agrobacterium tumefaciens* (LBA 4404). A = *Acc*I, CP = capsid protein, 8K = 8K protein, Hc = *Hic*II, P = *Pst*I, TER = terminator, Xb = *Xba*I, Xh = *Xho*I

Western blot analysis of coat protein expression. Total soluble proteins were isolated from young leaves of kanamycin-resistant potato plants, and from untransformed control plants. Leaf tissue (1 g) was extracted on ice with 3 ml of extraction buffer containing 0.3 M Tris-HCl, pH 8.3, 15 mM DTT, 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1 mM benzidine. Protein pellets were dissolved with sample buffer 0.5 M Tris-HCl, pH 6.8, 10% glycerol, 10% SDS w/v, 5% β-mercaptoethanol and 0.05% bromophenol blue and boiled at 100°C for 3 min before loading on the SDS-PAGE gel. After electrophoresis, the gel was incubated in transfer buffer (25 mM Tris HCl, pH 8.3, 192 mM glycine and 20% methanol). Polypeptides were transferred onto nitrocellulose membranes at a constant voltage of 30 V overnight. Membranes were washed with Tris-buffered saline (TBS, pH 8), and coated with 5% skimmed milk powder. Membranes were incubated overnight with rabbit anti-PVX CP polyclonal antibody conjugated to alkaline phosphatase (1:1000), then carefully washed with TBS, TBS + 0.05% (w/v) Tween-20 and TBS. Filters were incubated with the secondary antibodies (alkaline phosphatase-conjugated goat anti-rabbit IgG, from Bio-Rad, Melville, NY, USA)(1:1000) for 2 hr at 22°C with constant shaking. Filters were washed as described above and incubated for 10 min in developing buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl and 50 mM $MgCl_2$). Substrates NBT (35 µl) and BCIP (27 µl) in 10 ml of developing buffer was added and incubated in the dark. Color development was stopped by washing the filters with distilled water.

Inoculation of transgenic plants with PVX. Transgenic potato plants (from 4 cultivars, 11 clones) and untransformed controls were challenged with purified PVX. PVX was purified according the method of Eweida *et al.* (1990), and two concentrations of PVX, 5 µg/ml and 50 µg/ml, were used as inocula. Nine plants from each clone or control were used for the test. Five leaves of each plant were lightly dusted with carborundum (400 mesh), and each of these leaves was inoculated with 200 µl of virus solution. Inoculated plants were kept under greenhouse conditions (25°C and 16-hr photoperiod). Three weeks post inoculation, top and uninoculated leaves were picked from each inoculated plant and used for further local lesion and dot-blot hybridization tests.

Local lesion assay. Five to seven top uninoculated leaves from each inoculated potato plant were collected. One gram of leaf tissue was homogenized in 5 ml of 0.1 M phosphate buffer, pH 7.2, and the leaf sap was used as inocula on the local lesion host *Gomphrena globosa*. Opposite leaves of *G. globosa* were used as test and control, respectively. For each transgenic potato clone and control, at least 9 pairs of *G. globosa* leaves were inoculated. Inoculated plants were kept under greenhouse conditions as above for 4 to 6 days before the local lesions were counted.

Dot-blot hybridization assay. Two µl of random primers (hexamer oligodeoxyribonucleotides of 0.1 A_{260}/µl, Pharmacia) were annealed to 1 µg of PVX DNA (clone 117A linearized with *Hin*dIII) at 100°C for 3 min followed by quick cooling on ice. Five µl of α^{32}P-dATP (10 µCi), 10 µl of 2.5 × random primer buffer (500 mM Hepes, pH 6.6, 125 mM Tris, pH 8.0, 12.5 mM $MgCl_2$, 25 mM β-mercaptoethanol, 0.05 mM dTTP, 0.05 mM dGTP and 0.05 mM dCTP) and 1 unit of DNA polymerase I (Klenow fragment) were added. The reaction was performed at room temperature for 30 min and then at 37°C for 1.5 hr. The reaction was terminated by adding 1 µl of 0.5 mM EDTA, and the volume was adjusted to 100 µl with distilled water. The mixture was passed through a Sephadex G50 column. The probe was boiled for 3 min followed by quick cooling on ice just before use.

One gram of leaf tissue was homogenized in 2 ml extraction buffer containing 100 mM Tris-HCl, pH 8.0 and 0.1% of Triton X-100. Leaf sap was incubated for 30 minutes on ice and centrifuged at 16,000 g for 14 min at 4°C. The supernatant was collected and kept at -70°C for 60 min and then warmed to room temperature. Another centrifugation as above was carried out and the supernatant was collected for dot-blot hybridization.

Dot-blot hybridization assay was performed according to the method described by Nikolaeva *et al.* (1990). A series of dilutions of leaf samples was made, and 9 µl samples from each dilution were spotted onto a nitrocellulose filter and dried under an infrared lamp. Dry filters were floated on 10 × SSC/0.5% SDS at 56°C for 30 min. Filters were baked under vacuum at 80°C for 2 hr and prehybridized in 6 × SSC, 0.12% Ficoll (w/v), 0.12% polyvinyl-pyrrolidone (w/v), 0.12% BSA (w/v), 0.1% SDS and 100 µg/ml denatured herring sperm DNA at 60°C for 1 - 2 hours. The hybridization solution was replaced with a fresh solution containing denatured α^{32}P-labelled PVX DNA probes (20 ng/ml, 1.4×10^6 cpm/ml) and incubated at 60°C for 18 - 20 hr. Filters were washed twice in 2 × SSC/0.1% SDS for 20 min at room temperature and in 0.2 × SSC/0.1% SDS for 50 min at 56°C. Filters were exposed to X-ray film (Kodak XARS) at -70°C for 24 - 48 hr with an intensifying screen.

Field tests of transgenic potato plants for PVX resistance. Field experiments were carried out using virus-free, uniformly sized potato plants grown from tubers of four Russet Burbank and three Desireé transgenic potato clones. Untransformed virus-free Russet Burbank and Desireé potato plants were used as controls. The field was divided into 22 plots. Each plot contained 4 rows with 8 plants in each row. Plants from each transgenic clone and non-transgenic (control) cultivars were planted in 2 different plots (3 plots for each control). Plots were randomly distributed in the field. Four weeks after the plants were transferred into the field, mechanical inoculation was carried out using 1 µg/ml of purified PVX as inoculum. Alternating rows of transgenic and control plants were inoculated in each plot, and the other rows were left for yield measurements and comparison. Three leaves from each plant were taken before and after inoculation. Samples from both inoculated and uninoculated plants were taken weekly for 4 weeks post-inoculation and used to detect the level of PVX using dot-blot hybridization.

Results and discussion

In this report we described the introduction of resistance to PVX in four economically important potato cultivars, Russet Burbank, Shepody, Desirée and Bintje. A 1.3 Kbp fragment of PVX cDNA containing the 8K and CP genes and the 3' non-coding region of PVX genome was introduced into the expression vector Polyrok I (Fig.1). Both potato tubers and stem tissues were used for transformation. Our results (Table 1) show that the primary shoots (early regeneration) that regenerated from stem explants and the majority of those regenerated from tuber discs were not transgenic. Transformed shoots were recovered mostly from green callus proliferated from both stem explants and tuber discs. Shoots regenerated from potato tuber discs about one week after subculture onto MS31. Transformation efficiency of these shoots, although growing on 100 µg/ml kanamycin, was lower than that of the secondary shoots (late regeneration) that developed from the tubers after 2-3 subcultures in MS31 (Table 1). Apparently, the placement of tuber discs on medium containing growth regulators could break the bud dormancy and shoots emerged from already existing primodia. These shoots were unable

Table 1. Transformation/regeneration efficiency of different potato cultivars

Cultivar	Explant	Early Regeneration (1st culture)				Late Regeneration (2nd culture)			
		Culture efficiency[a]	Shoots (Total #)[b]	Rooted shoots (%)	CP (%)	Culture efficiency	Shoots (Total #)	Rooted shoots (%)	CP (%)
Desireé	TD[c]	20/100	100	70	10	40/80	90	90	50
Shepody	TD	13/100	15	50	ND	25/70	27	ND	ND
Shepody	S	30/95	32	0	0	34/70	40	95	35
Russet Burbank	TD	9/100	12	25	ND	20/75	22	95	25
Russet Burbank	S	20/100	22	0	0	20/65	26	95	25
Bintje	TD	5/100	6	ND	ND	15/50	ND	ND	25

[a] Culture efficiency = number of explants regenerated/number of explants cultured.
[b] Total number of shoots is higher than the number of regenerated explants because there was more than one shoot per inoculated explant. However, only one shoot per explant was used for rooting and further testings.
[c] TD: tuber disc; S: Stem; ND: Not determined; CP: Coat protein expression as determined by Western hybridization.

to root in medium containing 100 µg/ml kanamycin. After the primary shoots were excised, tuber discs formed callus, and new shoots developed. Although the efficiency of regeneration from stem explants was higher than from tubers, this explant source resulted in more escapes in both Shepody and Russet Burbank, based on their inability to root and survive on 100 µg/ml kanamycin. During the early culture period, 20-30 of the stem explants regenerated shoots. However, none of these shoots rooted in medium containing 100 µg/ml kanamycin. The secondary shoots, regenerated from callus which formed after removal of the primary shoots, successfully rooted on medium containing 100 µg/ml kanamycin. Both transformation protocols used in this work resulted in morphologically normal shoots.

The transformation attempts resulted in a total of 40 kanamycin-resistant plants. All transgenic plants were analyzed by Western blotting for the expression of capsid protein. Eleven lines were found to accumulate detectable amounts of PVX CP (Fig.2). The accumulated level of CP ranged between 0.05-0.2% of extracted total plant protein. Several plants showed bands corresponding to PVX CP. Analysis of PVX CP accumulation in different leaves of transformed plants

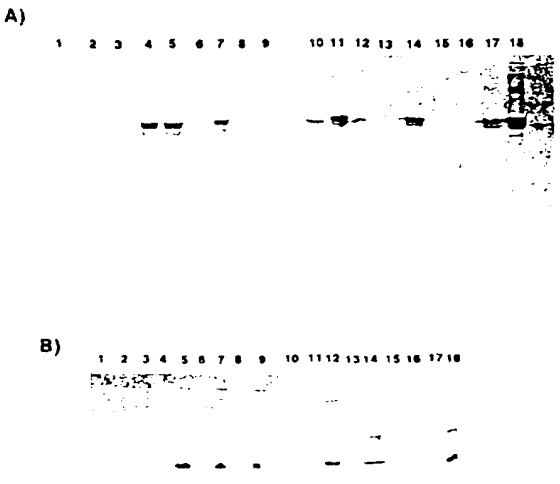


Fig.2. Western blot analysis of PVX CP expression in transgenic potato plants. Proteins (15 µg) were extracted from leaves, separated on a 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with rabbit anti-PVX CP polyclonal antibody conjugated to alkaline phosphatase.

A). Lane 1: 5 µg protein of control (untransformed) Russet Burbank; Lanes 2-10: transformed tubers of Russet Burbank giving rise to the following transgenic clones: 267, 278, 288 and 273 in lanes 4, 5, 7 and 10 respectively; Lanes 11-14: transformed stems of Shepody giving rise to two plants of clone 304 in both lanes 11 and 14; Lanes 15-17: transformed tubers of Russet Burbank giving rise to transgenic clone 286 in Lane 17; Other lanes were clones that showed no detectable CP. Lane 18 corresponds to 100 ng of PVX CP control.

B). Lane 1: control as in A; Lanes 2-11: transformed tubers of Desirée giving rise to transgenic clones 102, 35 and 25 in lanes 5, 7 and 9 respectively; Lanes 12 and 13: transformed tubers of Russet Burbank giving rise to clone 258 in lane 12; Lanes 14-17: transformed tubers of Bintje giving to transgenic clone 223 in lane 14. Other lanes were clones that showed no detectable CP. Lane 18 corresponds to 100 ng of PVX CP control.

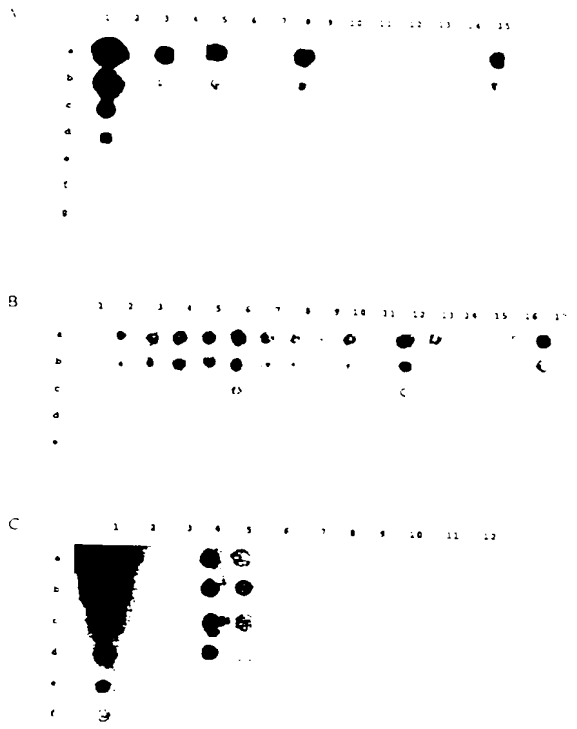


Fig.3. Dot-blot hybridization of PVX specific α^{32}P-labelled DNA probes to leaf extracts from potato plants inoculated with PVX in the greenhouse. A (5 µg/ml) and B (50 µg/ml) under greenhouse conditions and C (1 µg/ml) in the field

A) Purified PVX (a = 0.1 µg, b = 10 ng, c = 1 ng, d = 0.1 ng, e = 10 pg, f = 1 pg) and uninfected nontransformed potato (Shepody): lanes 1, 2; PVX infected nontransformed Bintje, Shepody and Russet Burbank: lanes 3, 5, 8; PVX infected transgenic clones Bintje 232, Shepody 304a,b, Russet Burbank 258, 267, 273, 278, 286 and 288: lanes 4, 6, 7, 9 - 14; PVX infected *Nicotiana tabacum* var. Samsun: lane 15; Dilution factors in lanes 2 - 15: a = 1/1, b = 1/50, c = 1/100, d = 1/200, e = 1/400, f = 1/800 and g = 1/1600

B). Uninfected nontransformed potato (Russet Burbank): lane 1; Inoculated nontransformed Bintje, Shepody, Desireé and Russet Burbank: lanes 2, 4, 6, 10; Inoculated transgenic clones Bintje 232, Shepody 304, Desireé 25, 35 and 102, Russet Burbank 258, 268, 273, 278, 286 and 288: lanes 3, 5, 7 - 9, 11 - 16; Purified PVX (a = 10 ng, b = 1 ng, c = 0.1 ng, d = 10 pg, e = 1 pg, f = 0.1 pg) lane 17; Dilution factors in lanes 1 - 16: a = 1/50, b = 1/100, c = 1/500, d = 1/1000, e = 1/2000

C) Purified PVX (a = 1 µg, b = 0.1 µg, c = 10 ng, d = 1 ng, e = 0.1 ng, f = 10 pg): lane 1; Uninfected nontransformed Russet Burbank and Desiree: lanes 2, 12; PVX inoculated nontransformed Russet Burbank and Desiree: lanes 4, 8; Inoculated transgenic Russet Burbank clones 267, 273, 286, 258 and Desiree clones 102, 35, and 25: lanes 3, 5 - 7, 9 -11; Dilution factors in lanes 2 - 12: a = 1/50, b = 1/100, c = 1/200, d = 1/400, e = 1/800, f = 1/1600

and in different plants originating from micropropagation of one original clone showed that the expression was independent of the leaf age (results not shown). Furthermore, re-propagated potatoes of the original Shepody 304 clone showed measurable amounts of CP. Since transgenic potato clones have both 8K and CP genes (Fig.1), the capsid protein present in these transgenic plants might be expressed by internal ribosome reinitiation (Morozov *et al.*, 1991). Potato plants transformed with the 8K protein of PVX and PVY CP genes showed, as expected, protection against PVY infection. However, when these transgenic plants were challenged with PVX, there was no protection against PVX, suggesting that the 8K protein does not play a role in the protection against PVX (Hefferon *et al.*, 1995, in preparation). Transgenic *Nicotiana benthamiana* plants expressing wild type movement proteins (13K and 7K) of white clover mosaic potexvirus (WClMV) did not confer resistance to WClMV infection, while only those transgenic plants expressing mutants of the 13K movement protein were resistant (Beck *et al.*, 1994). Consequently, the resistance of transgenic potato against PVX infection presented here is likely due to PVX capsid protein and not to the 8K protein. The PVX CP may also act as an avirulence determinant which can trigger the host resistance response to virus infection (Cruz and Baulcombe, 1993). A different mechnism which is based on the presence of untranslatable sense RNA for resistance in the potyvirus group was proposed by Lindbo *et al.* (1993) and Smith *et al.* (1994), where the resistance may be mediated by a cellular pathway, which may eliminate or down-regulate aberrant or overexpressed mRNAs. This observation seems to be unique to potyviruses.

Table 2. Local lesion assay showing the resistance of transgenic potato plants to PVX infection when challenged with 5 µg/ml PVX

Cultivar	Clone	Infectivity* (%)
Bintje	232	0
Shepody	304	0
Desiree	25	0
	35	0
	102	6.4 ± 0.4
Russet Burbank	258	0
	267	1.5 ± 1.4
	273	2.7 ± 0.2
	278	20.7 ± 3.1
	286	1.7 ± 1.4
	288	18.7 ± 1.3

* Infectivity (%) = number of local lesions (Test/Control) x 100. Nine plants from each transgenic clone were challenged with PVX. Five leaves of each infected plant were used for the local lesion assay. Nine pairs of *Gomphrena globosa* leaves from each sample were inoculated. Results are the average of 3 independent experiments and the numbers are shown with standard deviation.

After inoculation with the lower concentration of virus, 5 µg/ml, nontransformed potato plants showed high levels of virus accumulation two weeks after inoculation, while transgenic plants showed no virus accumulation after two weeks (data not shown), and absence or very little virus accumulation after three weeks, based on the local lesion assay and dot-blot hybridization (Table 2 and Fig.3). The extent of resistance to PVX infection among various transgenic clones was variable. Clones Bintje 232, Desirée 25 and 35, Shepody 304, Russet Burbank 258, 267 and 286 were resistant and showed little or no accumulation of PVX viral RNA in the upper uninoculated leaves three weeks after inoculation and were free of any visible symptoms. In contrast, clones of Russet Burbank 278 and 288 showed lower levels of resistance to PVX infection (Table 2) and accumulated higher levels of PVX viral RNA (Fig.3A). When challenged with the higher concentration of inoculum (50 µg/ml of purified PVX), all tested clones were positive for PVX after three weeks. In these conditions, virus accumulation relative to that of nontransformed control plants, varied between 25 and 100% three weeks post-inoculation (Fig.3B). This concentration of inoculum is 10 to 50 times higher than that used in other PVX resistance tests of transgenic potatoes (Lawson *et al.* 1990; Hoekema *et al.* 1991; Jongdijk *et al.* 1992).

Transgenic Russet Burbank (4 clones) and Desireé (3 clones) and control potato plants were grown in the field and mechanically inoculated with PVX. Four weeks post-inoculation, typical PVX symptoms, which include slight mosaic and mottling, were seen on young leaves of inoculated, untransformed potato plants and on some transgenic plants. Most of the transgenic clones showed no detectable PVX accumulation prior to inoculation, and there was little or no detectable PVX three weeks after inoculation (Fig.3C). However, PVX was detected in nontransformed Russet Burbank and Desireé plants after inoculation. Knowing that the susceptibility of the two cultivars (Russet Burbank and Desireé) to PVX is quite different, inoculated transgenic clones Russet Burbank 273 and Desireé 102 showed 57.1% and 16.6% of PVX accumulation, compared to their corresponding cultivar controls, respectively (Fig.3C).

In this paper we demonstrated that several transgenic potato cultivars are resistant to higher doses of PVX under greenhouse and field conditions. The use of PVX transgenic potato in the field can greatly delay PVX infection, and consequently reduce the virus accumulation in the plants and improve yield.

Acknowledgments. This work was supported in part by separate grants from the Ontario Ministry of Environment, the Ontario Ministry of Food and Agriculture and by an NSERC strategic grant to M G A.

References

Audy P, Palukaitis P, Slack SA, Zaitlin M (1994) Mol Plant-Microbe Interact 7:15-22
Beck AL, Van Dolleweerd CJ, Lough TJ, Balmori E, Voot DM, Andersen MT, O'Brien IEW, Forster RLS (1994) Proc Natl Acad Sci USA 91: 10310-10314
Bevan MW (1984) Nucl Acids Res 12: 8711-8721
Cruz SS, Baulcombe DC (1993) Mol Plant-Microbe Interact 6: 707-714
De Block M (1988) Theor Appl Genet 76: 767-774
Donson J, Kearney CM, Turpen TH, Khan IA, Kurath G, Turpen AM, Jones GE, Dawson WO, Lewandowski DJ (1993) Mol Plant-Microbe Interact 6: 635-642
Eweida M, Xu H, Singh RP, AbouHaidar MG (1990) Plant Pathol 39: 623-628
Fang G, Grumet R (1993) Mol Plant-Microbe Interact 6: 358-367
Farinelli L, Malnoë P (1993) Mol Plant-Microbe Interact 6: 284-292
Fitchen JH, Beachy RN (1993) Annu Rev Microbiol 47: 739-763
Fulton RW (1986) Annu Rev Phytopathol 24: 67-81
Gamborg OL, Miller RA, Ojima K (1968) Exp Cell Res 50: 151-158
Hemenway C, Fang RF, Kaniewski WK, Chua NH, Tumer NE (1988) EMBO J 7: 1273-1280
Hoekema A, Huisman MJ, Willink DP-L, Jongedijk E, van den Elzen P, Cornelissen BJC (1991) Plant Mol Biol 2: 183-192
Jongedijk E, de Schutter AAJM, Stolte T, van der Elzen PJM, Cornelissen BJG, (1992) Bio/Technology 10: 422-429
Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer NE (1990) Bio/Technology 8: 127-134
Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Plant Cell 5: 1749-1759
Matthews REF (1949) Annu Appl Biol 36: 460-474
Morozov SYu, Miroshnichenko NA, Solovyev AG, Fedorkin ON, Zellenina DA, Lukasheva LI, Karasev AV, Dolja VV, Atabekov JG (1991) J Gen Virol 72: 2039-2042
Murashige T, Skoog F (1962) Physiol Plant 15: 473-497
Nakajima M, Hayakawa, T, Nakamura, I, Suzuki, M (1993) J Gen Virol 74: 319-322
Nikolaeva OV, Morozov SYu, Zakhariev VM, Skryabin KG (1990) J Phytopathol 129: 283-290
Rubino L, Lupo R, Russo M (1993) Mol Plant-Microbe Interact 6: 729-734
Simth HA, Swaney SL, Parks TW, Wernsman EA, Dougherty WG (1994) Plant Cell 6: 1441-1453
Torrance L, Larkins AP, Butcher GW (1986) J Gen Virol 67: 57-67
Vance VB (1991) Virology 182: 486-494

Journal of General Virology (1997), 78, 1241–1246. Printed in Great Britain

SHORT COMMUNICATION

Pd. 1997-06
P. 1241-1246 : (6)

XP-002073446

Movement protein-derived resistance to triple gene block-containing plant viruses

P. Seppänen,[1] R. Puska,[1] J. Honkanen,[1] L. G. Tyulkina,[2] O. Fedorkin,[2] S. Yu. Morozov[2] and J. G. Atabekov[2]

[1] Department of Biological Research, Kemira Agro OY, Espoo Research Centre, 02271 Espoo, Finland
[2] Department of Virology, Moscow State University, Moscow 119899, Russia

Two mutant potato virus X (PVX) movement protein (MP) genes (m12K-Sal and m12K-Kpn) were obtained by inserting specific linkers at the boundary between the N-terminal hydrophobic and putative transmembrane segment, and the central invariant hydrophilic region of the respective 12 kDa, 12K, triple gene block (TGB) protein. Several transgenic potato lines which expressed m12K-Sal or m12K-Kpn to different degrees were resistant to infection by PVX, potato aucuba mosaic potexvirus and the carlaviruses potato virus M and S over a wide range of inoculum concentrations (3–300 μg/ml). However, they were not resistant to potato virus Y, which lacks a TGB protein. We suggest that the resistance of m12K-Sal and m12K-Kpn transgenic potato lines is MP-derived and not RNA-mediated.

The efficiency of cell-to-cell virus movement is important in determining pathogenicity, virulence and, in some cases, the host range of a plant virus (reviewed by Atabekov & Taliansky, 1990; Maule, 1991). When the efficiency of the transport function and the rate of virus movement are reduced, the plant acquires a certain level of resistance to virus infection.

Production of dysfunctional or partially active movement proteins (MP) in transgenic plants is assumed to confer resistance to the wild-type (wt) virus by competition between wt virus-coded MP and the preformed modified MP (mMP). In support of this assumption, it has been reported that transgenic tobacco plants which produce a non-functional MP of tobacco mosaic virus (TMV) acquire resistance to TMV infection (Malyshenko *et al.*, 1993; Lapidot *et al.*, 1993). In addition, transgenic plants expressing non-functional TMV MP were resistant to several distantly related or unrelated viruses (Lapidot *et al.*, 1993; Cooper *et al.*, 1995). These results suggest that there are conserved functions among the MP of different plant viruses, despite an apparent lack of sequence similarity.

Unlike tobamoviruses and many other viruses which have a single MP gene, a module of three partially overlapping genes known as the triple gene block (TGB) was found in the genomes of potexviruses, carlaviruses, hordeiviruses and some furo-like viruses (Morozov *et al.*, 1989; Rupasov *et al.*, 1989; Scott *et al.*, 1994). Mutations in each of the TGB genes have been shown to inhibit the virus transport function (Petty & Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Angell *et al.*, 1996). The first ORF of the potato virus X (PVX) TGB encodes a protein of molecular mass 25 kDa (25K) (Fig. 1*a*) (Skryabin *et al.*, 1988). The 12K and 8K proteins encoded by the second and third ORFs of PVX TGB (Fig. 1*a*) and their counterparts in other viruses, possess hydrophobic domains (Fig. 1*b*) and were found to associate with a membrane-enriched fraction *in vitro* and *in vivo* (Morozov *et al.*, 1990; Richards & Tamada, 1992; Donald *et al.*, 1995).

Recently, it was shown that *Nicotiana benthamiana* plants which express a modified 13K MP, encoded by the central TGB ORF of white clover mosaic potexvirus (WClMV) (Fig. 1*b*), were resistant to systemic infection with WClMV, two other potexviruses and potato virus S (PVS) (Beck *et al.*, 1994). In this paper, we show that expression in transgenic potato plants of the PVX 12K MP gene, specifically modified at the region encoding the boundary between the hydrophobic and hydrophilic segments, confers MP-derived resistance to PVX, potato aucuba mosaic potexvirus (PAMV) and the carlaviruses potato virus M (PVM) and PVS. Movement of these viruses within the inoculated leaves was blocked. However, the plants were susceptible to potato virus Y (PVY).

A portion of the PVX genome including the 12K and 8K genes (Fig. 1*a*) was amplified and cloned into the T7-transcription vector pBS (Stratagene) to give pXT7-12, as described (Morozov *et al.*, 1991). pRT12WT was constructed by cutting pXT7-12 at an *Eco*RI site artificially introduced upstream of the 12K gene and a *Hin*dIII site (position 5685 in the PVX genome) and inserting the isolated fragment (containing the wt TGB 12K and 8K protein genes) into *Eco*RI–*Sma*I-cleaved pRT101 (Töpfer *et al.*, 1987). pUC12WT

Author for correspondence: Joseph Atabekov
Fax + 7 095 938 06 01. e-mail atabekov@virus.genebee.msu.su

0001-4478 © 1997 SGM

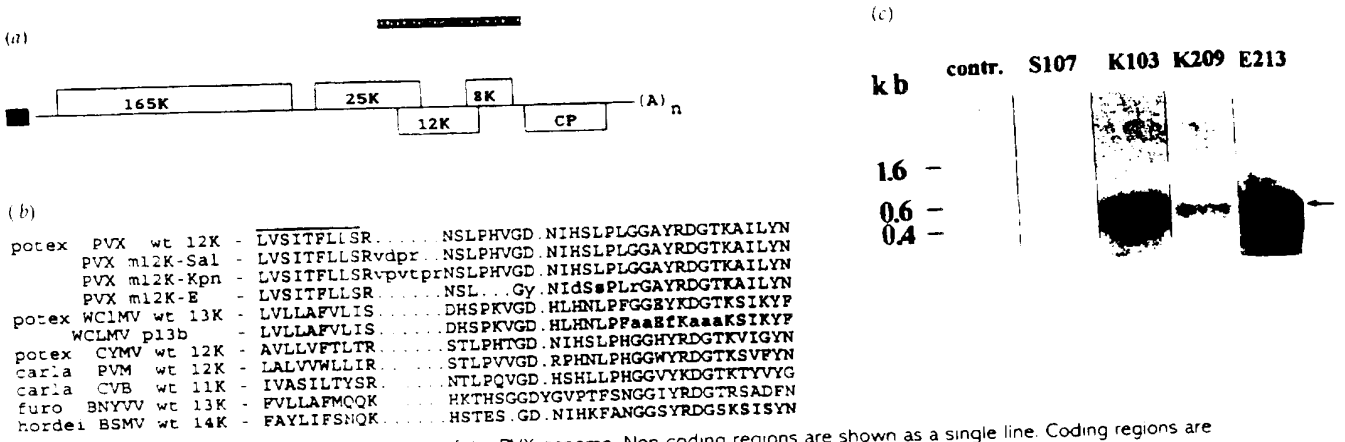


Fig. 1. (*a*) Schematic representation of the PVX genome. Non-coding regions are shown as a single line. Coding regions are shown by open boxes and indicated by the molecular mass (K, kDa) of their products or as coat protein (CP). The filled box indicates the 5′ cap structure. The thick line above shows the portion of the PVX genome cloned in the transgenes of the S-, K- and E-series. (*b*) Alignment of conserved hydrophilic regions of the proteins coded by the central TGB ORF of PVX, white clover mosaic (WClMV) and clover yellow mosaic (CYMV) potexviruses, potato virus M (PVM) and chrysanthemum virus B (CVB) carlaviruses, beet necrotic yellow vein furovirus (BNYVV) and barley stripe mosaic hordeivirus (BSMV). The positions of amino acid substitutions in the 13K MP of the WClMV mutant p13b (Beck *et al*., 1994) and the PVX mutant m12K-E, and the insertions in PVX mutants m12K-Sal and m12K-Kpn are shown in lower-case letters. Part of the N-terminal hydrophobic segment is overlined above the PVX sequence. (*c*) Northern blot analysis of total RNA extracted from leaves of untransformed potato (cv. Pito) as a control and mMP$^+$ transgenic lines S107, K103, K209 and E213. Molecular masses are marked in kb. The arrow indicates the predicted position of 12K/8K mRNA expressed from integrated chimaeric pBIN sequences. The probe was ^{32}P-labelled pUC12WT.

was constructed by subcloning a *Xho*I–*Bam*HI fragment from pRT12WT into *Sal*I–*Bam*HI-cleaved pUC19.

To create the m12K-Sal and m12K-Kpn mutants of the 12K MP several codons were inserted (in frame) into the central hydrophilic domain of the 12K protein gene (Fig. 1*b*). A 12 nt insertion (mutant m12K-Sal) and an 18 nt insertion (mutant m12K-Kpn) in the 12K protein gene were created by inserting a modified *Sal*I linker (octamer) and a *Kpn*I linker (14-mer), respectively, into the filled *Xba*I site (position 5249 in the PVX genome) of pUC12WT, to give pUC-Sal and pUC-Kpn. pRT-Sal and pRT-Kpn were constructed by inserting an *Eco*RI–*Bam*HI fragment of pUC-Sal and pUC-Kpn, respectively, into pRT101.

The mutant m12K-E (Fig. 1*b*) used in this study was constructed by PCR-mediated mutagenesis using pUC12WT DNA as template and two primers: 12mod-*Xba*, 5′ GCTTtctagaAATAGTTTAGGTTATAACATTGACAGTTCACC ACACAGAGGA 3′, and the universal sequencing primer. The primer 12mod-*Xba* corresponds to an internal region of the PVX 12K gene including an authentic *Xba*I site (shown in lower-case) and a 9 nt deletion and four single base pair changes compared to the wild-type PVX RNA sequence (positions 5245–5305). The resulting PCR fragment was cleaved with *Xba*I and *Bam*HI and cloned into pUC12WT, to give pUC-E. pRT-E was constructed by inserting the *Eco*RI–*Bam*HI fragment of pUC-E into pRT101. The 12K gene in pUC-E was sequenced to confirm the modified gene. Plant expression cassettes were constructed in the binary vector pBIN19 (Bevan, 1984) by excision of *Hin*dIII fragments from the pRT-based plasmids (pRT-Sal, pRT-Kpn and pRT-E) and insertion into *Hin*dIII-cut pBIN19.

pBIN19 plasmids containing the m12K-Sal, m12K-Kpn or m12K-E genes were conjugated into *Agrobacterium tumefaciens* pGV2260, a non-oncogenic helper strain. Pieces of potato stem (*Solanum tuberosum* cv. Pito) were used for *Agrobacterium*-mediated transformation as described by Truve *et al*. (1993). Transgenic lines with the modified m12K-Sal, m12K-Kpn and m12K-E genes were referred to as S-, K- and E-series lines, respectively.

Of 44 transformed potato lines 15 S-, 12 K- and 10 E-series lines were selected for further analyses by Northern and/or Southern blotting. In Northern blots, several independent lines of each series showed the presence of 12K and 8K mRNA (mMP$^+$) of the expected size. Fig. 1(*c*) shows Northern blot analyses of some lines used for further testing of virus resistance. Some lines showed no 12K or 8K RNA expression (data not shown) and were not tested for resistance. Significant differences in RNA expression levels were also noticed between different mMP$^+$ clones (Fig. 1*c* and data not shown).

Three-week-old plants obtained from transformed tissue explants, with five to six true leaves and already containing leaflets, were used for challenge virus inoculations (three to six leaves were inoculated per plant). Three inoculum dilutions (300, 30 and 3 μg/ml) of PVX (Russian strain) were used for the infectivity tests. Leaf tips (about one-third of the leaf) were inoculated mechanically with 20–30 μl inoculum. Virus accumulation was tested by DAS–ELISA (Clark & Adams, 1977) 10 days post-inoculation (p.i.) in apical inoculated (AI), non-

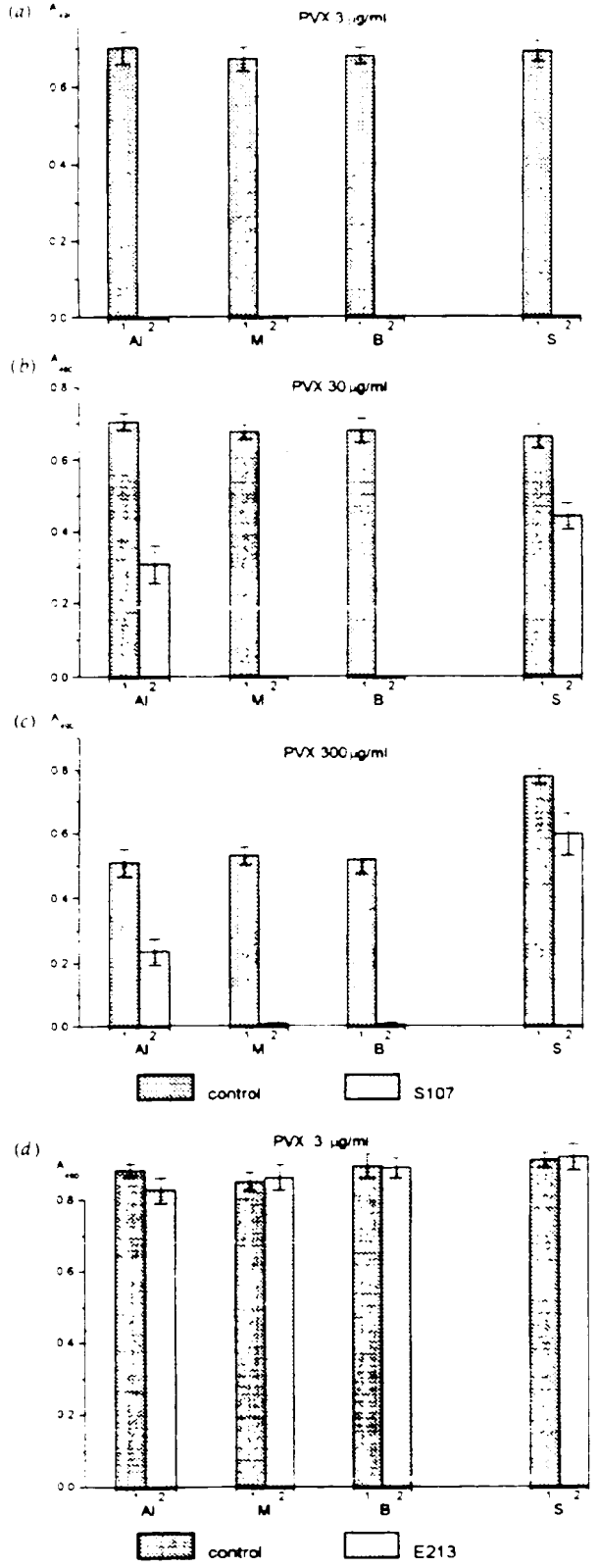


Fig. 2. Reaction of potato plants expressing the modified 12K MP PVX gene to PVX. (*a–c*) Resistance of plants transgenic for the m12K-Sac gene (line S107) to PVX at three inoculum concentrations (3, 30 or 300 µg/ml). Analogous data (not shown) were obtained with the m12K-Kpn gene. (*d*) Susceptibility of plants transgenic for the m12K-E gene (line E213) to PVX inoculated at 3 µg/ml. A_{405} represents the DAS–ELISA absorbance values with peroxidase-conjugated antibodies. Virus was detected by DAS–ELISA in pooled samples from five to six inoculated leaves of each plant and data were averaged for five to six separate plants 10 days p.i. (for inoculated leaves) and 30 days p.i. (for systemic leaves). Standard error bars are presented. Al, Apical inoculated; M, middle non-inoculated; B, basal non-inoculated sections of the same leaf; S, systemically infected leaves. Columns 1, control (MP$^+$) plants. Columns 2, MP$^-$ transgenics of lines S107 (*a–c*) or E213 (*d*) expressing a mutated 12K MP gene.

inoculated middle (M) and basal (B) sections of the challenged leaf, as well as in non-inoculated (systemically infected) leaves (S) at 30 days p.i. All leaf sections from one plant were pooled for further testing by DAS–ELISA.

To check for virus resistance, selected lines with different levels of PVX-specific mRNA expression (Fig. 1*c*) in the S- (S101, S102, S107, S113, S115 and S119), K- (K103, K106, K107, K207, K209 and K210) and E-series (E203, E204, E213 and E215) plants, as well as non-transgenic control plants, were inoculated with PVX and other viruses. Plants of line S107 (Fig. 2), K209 (Fig. 3*d*), K103, K106, K107, K207, S101, S102, S113 and S115 (data not shown), which expressed modified 12K genes (mMP$^-$), were completely resistant to PVX infection at an inoculum concentration of 3 µg/ml. Consequently, both cell-to-cell and systemic movement of PVX were blocked in transgenic mMP$^-$ plants of the K- and S-series. Importantly, there was no correlation between 12K transgene mRNA accumulation and resistance (that is high levels of resistance were found both in high and low mRNA expressing lines). We propose that even low level m12K expression supports the resistant phenotype.

At higher PVX inoculum concentrations (30–300 µg/ml), the accumulation of PVX in Al leaf sections of mMP$^-$ plants was reduced in comparison to control MP$^-$ (Fig. 2*b*, *c*); however, the level of resistance was considerably lower than that with an inoculum concentration of 3 µg/ml. It should be noted that when virus accumulated to a certain level in the Al section of the leaf, systemic spread of infection occurred even in the absence of virus accumulation in the M and B sections (Fig. 2*b*, *c*).

mMP$^-$ plants of the m12K-E series were exceptional. Despite the high level of PVX-specific RNA expression detected by Northern blotting, no resistance to PVX infection was detected in all four lines tested: E213 (Fig. 2*d*), E203, E204 and E215 (data not shown). The reason for the inability of this 12K MP modification to confer resistance to PVX is not clear.

PAMV potexvirus is distantly related to PVX, whereas PVS and PVM are members of the genus *Carlavirus*. Although these viruses encode TGB proteins, their genomes share little nucleotide sequence similarity with the PVX genome (Xu *et al.*, 1994; Rupasov *et al.*, 1989). Transgenic potato plants (mMP$^-$ line K209) transformed with the mutated PVX 12K and wt 8K

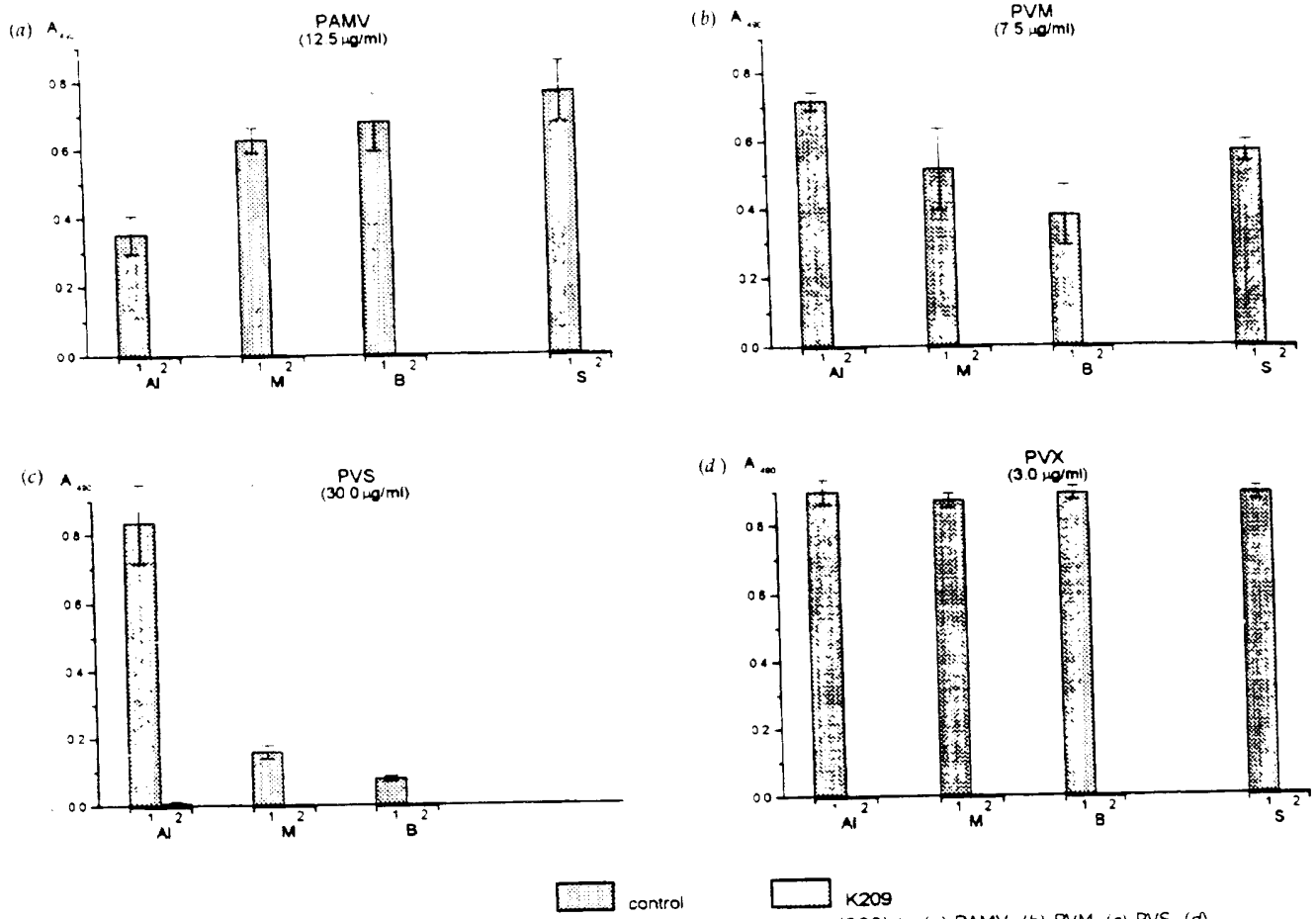


Fig. 3. Resistance of potato plants expressing a modified 12K MP PVX gene (line K209) to (*a*) PAMV, (*b*) PVM, (c) PVS, (*d*) PVX. Bars show DAS–ELISA data for the accumulation of each virus in transgenic PVX mMP$^+$ plants or control plants inoculated with 12·5 µg/ml PAMV, 7·5 µg/ml PVM, 30 µg/ml PVS or 3 µg/ml PVX, respectively. Abbreviations are as for Fig. 2. Columns 1, control (MP$^-$) plants; Columns 2, Mmp$^+$ transgenics of line K209 expressing a mutated 12K MP gene.

genes were inoculated with PAMV, PVS and PVM by the procedure described above. Fig. 3 (*a–c*) shows that transgenic plants were resistant to all three viruses, at least at the inoculum concentrations used in our experiments.

It can be concluded that both cell-to-cell movement and systemic spread of PAMV, PVS and PVM were blocked in the K209 transgenic plants. In a separate series of experiments (data not shown), we found that transgenic plants of lines K103 and S107 were also resistant to PAMV, PVS and PVM.

To determine whether the resistance was also manifest against viruses lacking a TGB gene, transgenic K- and S-series potato plants were inoculated with PVY^0. No significant differences in the levels of PVY^0 accumulation in inoculated or in systemically infected leaves were observed in mMP$^+$ transgenic plants compared with control (MP$^-$) potato plants (data not shown).

Transgenic mMP$^+$ *N. benthamiana* plants (Beck *et al.*, 1994) which expressed the mutated 13K WClMV MP (Fig. 1*b*) were found to be resistant to systemic infection with WClMV, PVX, PVS and narcissus mosaic potexviruses, but were susceptible to TMV, lacking a TGB protein. These observations suggested that expression of the modified 12K/13K MP gene of a TGB was able to protect transgenic plants against a range of viruses possessing a TGB protein; however, there was no protection against viruses lacking a TGB protein.

We suggest that the resistance of mMP$^+$ plants (S- and K-series) was due to interference between the modified (presumably partially functional) 12K MP and that of the challenging virus, rather than to a sense-RNA-mediated effect (Smith *et al.*, 1994; English *et al.* 1996). Unfortunately, this particular point was not discussed by Beck *et al.* (1994).

Several lines of evidence strongly support this theory. Firstly, the susceptibility of four lines of E-series plants to PVX (Fig. 2*d*) shows that, despite the almost complete sequence homology between wt PVX RNA and the RNA produced by the m12K-E transgene, there was no resistance to PVX. This point is also supported by our unpublished data on the complete lack of resistance to PVX in *N. benthamiana* and *N.*

tabacum plants expressing wt PVX 12K and 8K protein genes. Secondly, a high level of PVX accumulation (over 70% in comparison with wt plants) in non-inoculated systemic leaves of transgenic potato (Fig. 2*b*, *c*) is not consistent with the blockage of RNA replication or 'recovery' phenotype provoked by an RNA-mediated mechanism. Finally, it is unlikely that resistance of mMP$^+$ plants to four different viruses (PVX, PAMV, PVS and PVM) with low nucleotide sequence identity (30–50%) in the TGB region was caused by the highly sequence-specific nature of RNA-mediated resistance (English *et al.*, 1996). Additionally, in the most conserved hydrophilic region, PVX displays 68–70% amino acid sequence identity and only 62–63% nucleotide sequence identity with PAMV, PVS and PVM. Also, in the m12K-E transgene there is a $\geqslant$ 90% nucleotide sequence identity to wt in the hydrophilic region yet no resistance was observed in plants of four independent lines. These facts unequivocally argue against an RNA-mediated mechanism of resistance. It is probable that the MP encoded by the central gene of the TGB of different viruses share a common function not present in the MP of TGB-lacking viruses. Therefore, the modified 12K genes (m12K-Sal and m12K-Kpn) act in mMP$^+$ plants as dominant negative mutants affecting different TGB-possessing viruses.

We thank Dr A. F. Bobkova for DAS–ELISA tests and Dr A. G. Solovyev for some gene constructs. We also thank Dr T. Teeri for the *Agrobacterium* vector constructs and K. Koivunen, A. Kerttula and A. Aaltonen for transformation and technical assistance.

References

Angell, C. H., Davies, C. & Baulcombe, D. C. (1996). Cell-to-cell movement of potato virus X is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of *Nicotiana clevelandii*. *Virology* **216**, 197–201.

Atabekov, J. G. & Taliansky, M. E. (1990). Expression of a plant virus-coded transport function by different viral genomes. *Advances in Virus Research* **38**, 201–247.

Beck, D. L., Guilford, P. J., Voot, D. M., Andersen, M. T. & Forster, R. L. S. (1991). Triple block proteins of clover mosaic potexvirus are required for transport. *Virology* **183**, 695–702.

Beck, D. L., Van Dolleweerd, C. J., Lough, T. J., Balmori, E., Voot, D. M., Andersen, M. T., O'Brien, I. E. W. & Forster, R. L. S. (1994). Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block. *Proceedings of the National Academy of Sciences, USA* **91**, 10310–10314.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12**, 8711–8721.

Clark, M. F. & Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475–483.

Cooper, B., Lapidot, M., Heick, J. A., Dodds, J. A. & Beachy, R. N. (1995). A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility. *Virology* **206**, 307–313.

Donald, R. G. K., Petty, I. T. D., Zhou, H. & Jackson, A. O. (1995). Properties of genes influencing barley stripe mosaic virus movement phenotypes. In *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance. Proceedings of the Fifth International Symposium*, pp. 115–150. Edited by D. D. Bills and others. Singapore: World Scientific.

English, J. J., Mueller, E. & Baulcombe, D. C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 179–188.

Gilmer, D., Bouzoubaa, S., Hein, A., Guilley, H., Richards, K. & Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3′ proximal genes located on RNA 2. *Virology* **189**, 40–47.

Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J. & Beachy, R. N. (1993). A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant Journal* **4**, 959–970.

Malyshenko, S. I., Kondakova, O. A., Nazarova, Ju. V., Kaplan, I. B., Taliansky, M. E. & Atabekov, J. G. (1993). Reduction of tobacco mosaic virus accumulation in transgenic plants producing non-functional viral transport proteins. *Journal of General Virology* **74**, 1149–1156.

Maule, A. (1991). Virus movement in infected plants. *Critical Reviews in Plant Sciences* **9**, 457–473.

Morozov, S. Yu., Dolja, V. V. & Atabekov, J. G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *Journal of Molecular Evolution* **29**, 52–62.

Morozov, S. Yu., Miroshnichenko, N. A., Zelenina, D. A., Fedorkin, O. N., Solovyev., A. G., Lukasheva, L. I. & Atabekov, J. G. (1990). Expression of RNA transcripts of potato virus X full-length and subgenomic RNAs. *Biochimie* **72**, 677–684.

Morozov, S. Yu., Miroshnichenko, N. A., Fedorkin, O. N., Solovyev, A. G., Zelenina, D. A., Lukasheva, L. I., Karasev, A. V., Dolja, V. V. & Atabekov, J. G. (1991). Expression strategy of the potato virus X triple gene block. *Journal of General Virology* **72**, 2039–2042.

Petty, I. T. D. & Jackson, A. O. (1990). Mutational analysis of barley stripe mosaic virus RNA β. *Virology* **179**, 712–718.

Richards, K. E. & Tamada, T. (1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annual Review of Phytopathology* **30**, 291–313.

Rupasov, V. V., Morozov, S. Yu., Kanyuka, K. V. & Zavriev, S. K. (1989). Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. *Journal of General Virology* **70**, 1861–1869.

Scott, K. P., Kashiwazaki, S., Reavy, B. & Harrison, B. D. (1994). The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. *Journal of General Virology* **75**, 3561–3568.

Skryabin, K. G., Morozov, S. Yu., Kraev, A. S., Rozanov, M. N., Chernov, B. K., Lukasheva, L. I. & Atabekov, J. G. (1988). Conserved and variable elements in RNA genomes of potexviruses. *FEBS Letters* **240**, 33–40.

Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A. & Dougherty, W. G. (1994). Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation and fate of nonessential RNAs. *Plant Cell* **8**, 1444–1453.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. & Steinbiss, H.-H. (1987). A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Research* **15**, 5890.

Truve, E., Aaspollu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T., Kelve, M., Seppanen, P. & Saarma, M. (1993). Transgenic

potato plants expressing mammalian 2′–5′ oligoadenylate synthetase are protected from potato virus X infection under field conditions. *Bio/Technology* **11**, 1048–1052.

Xu, H., Leclerc, D., Leung, B. & AbouHaidar, M. G. (1994). The entire nucleotide sequence and genomic organization of potato aucuba mos potexvirus. *Archives of Virology* **135**, 461–469.

Received 21 October 1996; Accepted 14 January 1997

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, A01H 5/00 // C07K 14/08

A1

(11) International Publication Number: WO 98/07875

(43) International Publication Date: 26 February 1998 (26.02.98)

(21) International Application Number: PCT/BE97/00092

(22) International Filing Date: 18 August 1997 (18.08.97)

(30) Priority Data:
96870106.0 19 August 1996 (19.08.96) EP
(34) Countries for which the regional or international application was filed. BE et al.

(71) Applicant (for all designated States except US): SES EUROPE N.V./S.A. [BE/BE]; Industriepark 15, B-3300 Tienen (BE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): GUILLEY, Hubert [FR/FR]; 32, rue de l'Herbe, F-67370 Berstett (FR). JONAR, Gérard [FR/FR]; 9, quai de Chanoine Winterer, F-67000 Strasbourg (FR). RICHARDS, Ken [FR/FR]; 2, rue Principale, F-67370 Pfulgriesheim (FR). BOUZOUBAA, Salah [FR/FR]; 11, rue de Berstett, F-67200 Strasbourg (FR). BLEYKASTEN-GROSSHANS, Claudine [FR/FR]; 9, rue du Renard Préchant, F-67000 Strasbourg (FR). WEYENS, Guy [BE/BE]; Dwersbos 25, B-1650 Beersel (BE). LEFEBVRE, Marc [BE/BE]; Rue de la Chapelle Stevenaert, 75, B-1370 Jodoigne (BE).

(74) Agents: VAN MALDEREN, Eric et al., Office Van Malderen, Place Reine Fabiola 6/1, B-1083 Brussels (BE).

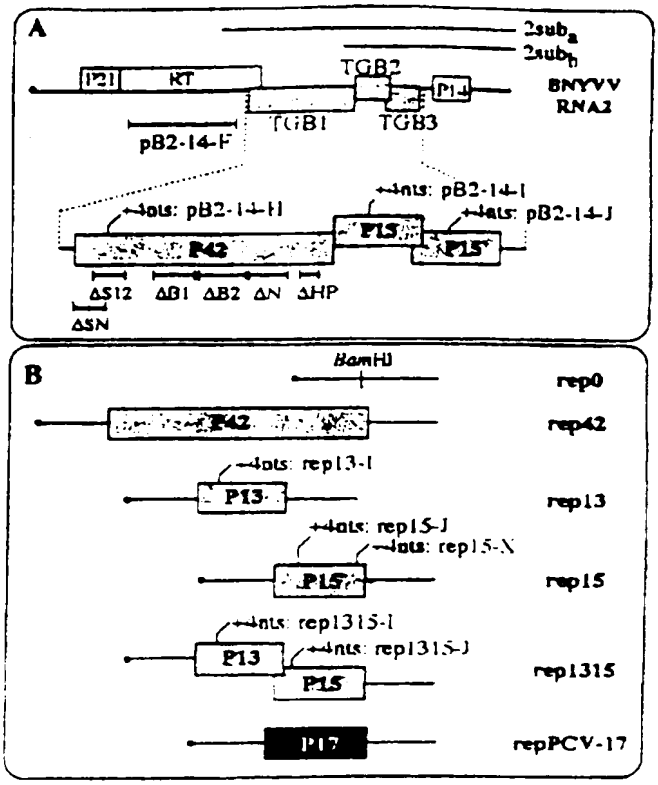
(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, DE (Utility model), EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR INDUCING VIRAL RESISTANCE INTO A PLANT

(57) Abstract

The present invention concerns a method for inducing resistance to a virus comprising a TGB3 sequence with the proviso that it is not the potato virus X, into a cell plant or a plant, comprising the following steps: preparing a nucleic acid construct comprising a nucleic acid sequence corresponding to at least 70 % of the nucleic acid sequence of TGB3 of said virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant, transforming a plant cell with the nucleic acid construct, and possibly regenerating a transgenic plant from the transformed plant cell. The present invention is also related to the plant obtained.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHOD FOR INDUCING VIRAL RESISTANCE INTO A PLANT

Field of the invention

The present invention is related to a method for inducing viral resistance into a cell and plant, especially BNYVV-resistance into a sugar beet cell and plant and the viral resistant cell and plant obtained.

Background of the invention and state of the art

The widespread viral disease of the sugar beet plant (*Beta vulgaris*) called Rhizomania is caused by a furovirus, the beet necrotic yellow vein virus (BNYVV) (23, 24) which is transmitted to the root of the beet by the soilborne fungus *Polymyxa betae* (25).

The disease affects significantly acreages of the area where the sugar beet plant is grown for industrial use in Europe, USA and Japan and is still in extension in several places in Western Europe (26, 27). As there exists no practical method to effectively control the spread of the virus at a large scale by chemical or physical means (28), neither in the plants nor in the soil, the main focus has been to identify natural sources of resistance within the sugar beet germplasm and to develop by breeding, varieties of sugar plants beet expressing the resistance

genes. A variety of such tolerance genes to the virus has been identified and, some have been successfully used in the breeding of commercial sugar beet varieties (29, 30, 31).

Only the use of BNYVV-resistant or tolerant varieties will enable farmers to grow sugar beet plants in BNYVV-infected areas where sugar beet plant is an essential component of the crop rotation and contributes significantly to the grower's income.

A number of detailed studies have shown that a difference in susceptibility to the BNYVV-infection among sugar beet genotypes or varieties, generally reflect difference in the diffusion or translocation of the virus in the root tissues (32).

However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm (33), would provide distinct mechanisms of resistance. Such a situation would represent a more manageable situation to design long lasting BNYVV-resistance strategies.

Since 1986, number of reports and publications have described the use of isolated viral gene sequences expressed in plant to confer a high level of tolerance against the virus or even to confer a broad spectrum type of resistance against a number of related viruses (34, 35, 36). One of the most documented viral resistance strategy based on genetic engineering, in many cultivated species such as potato, squash, cucumber or tomato, is the use of the viral gene sequence encoding the coat-protein of the target virus (37) which under the control of plant regulatory elements, will be expressed in

the plant.

However in the case of the coat-protein mediated resistance, the expression of a certain level of resistance in the transgenic plant might be attributed to different mechanisms such as RNA co-suppression and not necessarily to the production of the protein sequence.

In general, the virus sequence will be transformed in an appropriated cell or a tissue culture of the plant species using an *Agrobacterium* mediated transformation system or a direct gene transfer method according to the constraints of the tissue culture or cell culture method which can be successfully applied in a given species. A whole plant will be regenerated and the expression of the transgene will be characterized.

Though sugar beet is known as a recalcitrant species in cell culture, limiting the extent of practical genetic engineering applications in that species, there are number of isolated reports of successful transformation and regeneration of whole plants (38). A few examples of engineering tolerance to the BNYVV by transforming and expressing the BNYVV coat-protein sequence in the sugar beet genome have also been published (39, WO91/13159) though they rarely report data on whole functional transgenic sugar beet plants (40). In particular, reports show limited data on the level of resistance observed in infected conditions with transgenic sugar beet plants transformed with a gene encoding a BNYVV coat-protein sequence (41, 42).

A complete technology package including a sugar beet transformation method and the use of the expression of the BNYVV coat-protein sequence as resistance source in the transgenic sugar beet plant obtained by said

transformation method has been described in the Patent Application WO91/13159.

Based on the information published, it can not be concluded that the coat-protein mediated resistance mechanism provides any potential for conferring to the sugar beet plant a total immunity to the BNYVV-infection by inhibiting completely the virus multiplication and diffusion mechanisms. To identify a resistance mechanism which enables to block significantly the spread of the virus at the early stage of the infection process would be a major criteria of success to develop such a transgenic resistance, in addition to the fact that even a level of resistance comparable to those known from the genes of resistance identified within the sugar beet germplasm would diversify the mechanisms of resistance available.

Because the disease is shown to expand in many countries or areas, at a speed depending upon the combination of numerous local environmental and agricultural factors, there is a major interest to diversify the sources of genetic resistance mechanisms which may, alone or in combination, confer a stable and long lasting resistance strategy in the current and future varieties of sugar beet plants which are grown for industrial use.

The publication of Xu H. et al. (Plant Cell Report, Vol. 15, pp. 91-96 (1995)) describes genetically engineering resistance construct to potato virus X in four commercial potato cultivars. However, said document states that transgenic potato clones which have included the 8KG gene (the TGB3 construct). However, when these transgenic plants were challenged with PVX, there was no protection against PVX suggesting that the OK protein does not play a

role in the protection against PVX.

Aims of the invention

The present invention aims to provide a new method for introducing various viral resistances into a cell and a plant and the viral resistant cell and plant obtained.

A main aim of the invention is to provide a new method for introducing BNYVV resistance into a cell and a plant and the BNYVV-resistant cell and plant, in particular a sugar beet cell and plant (*Beta vulgaris ssp.*), obtained.

Summary of the invention

The present invention provides the use of an alternative sequence of plant virus, especially the BNYVV, to obtain a high degree of tolerance to the viral infection, in particular to ensure a rapid and total blocking of virus multiplication and diffusion mechanisms in a plant, especially in the sugar beet plant (*Beta vulgaris*), including fodder beet, Swiss Whard and table beet, which may also be subject to this viral infection. Expression of the resistance will be obtained in transgenic cell and plant, especially sugar beet cells and plants produced by the transformation method subject to the Patent Application WO95/10178 or by other transformation methods based on *Agrobacterium tumefaciens* or direct gene transfer. Because of its high efficiency, the transformation method as described in WO95/10178 enables the production of large numbers of transformated plants, especially sugar beet plants, and will be preferred to develop transgenic plants which may be analysed and characterized for their level of

viral resistance, especially BNYVV Resistance, including their field evaluation.

The genome of beet necrotic yellow vein furovirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4 and 5) are implicated in vector-mediated infection of sugar beet (*Beta vulgaris*) roots (1). Cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB) (2), which encode, in order, the viral proteins P42, P13 and P15 (gene products are designated by their calculated M_r in kilodalton (3).

In the following description, the TGB genes and the corresponding proteins will be identified by the following terms : TGB1, TGB2, TGB3 or by their encoded viral protein number P42, P13 and P15. TGB counterparts are present in other furoviruses (4, 5), and in potex-, carla- and hordeiviruses (6).

In the table 1 are represented viruses having a TGB3 sequence, the molecular weight of TGB3 of said viruses, their host and references.

Table 1

Virus	Size of TGB3	Host	Reference
Apple stem pitting virus	8 kDa	apple	Jelkman, *J. Gen. Virol.* 75, 1535-1542 (1994)
Blueberry scorch virus	7 kDa	blueberry	Cavileer et al., *J. Gen. Virol.* 75, 711-720 (1994)
Potato virus M	7 kDa	potato	Zavriev et al., *J. Gen. Virol.* 72, 9-14 (1991)
White clover mosaic virus	8 kDa	clover	Forster et al., *Nucl. Acids Res.* 16, 291-303 (1988)
Cymbidium mosaic virus	10 kDa	orchid	Neo et al., *Plant Mol. Biol.* 18, 1027-1029 (1992)
Barley stripe mosaic virus	17 kDa	barley	Gustafson et al., *Nucl. Acids Res.* 14, 3895-3909 (1986)
Potato mop top virus	21 kDa	potato	Scott et al., *J. Gen. Virol.* 75, 3561-3568 (1994)
Peanut clump virus	17 kDa	peanut	Herzog et al., *J. Gen. Virol.* 75, 3147-3155 (1994)
Beet soil-borne virus	22 kDa	sugar beet	Koenig et al., *Virology* 216, 202-207 (1996)

The Inventors propose herewith a new method for providing resistance to plant viruses into a plant by blocking virus multiplication and diffusion mechanisms into

said plant, especially into its root tissue. In order to demonstrate said resistance, the Inventors describe hereafter the effect of the overexpression of TGB sequences alone or in combination upon BNYVV multiplication and diffusion mechanism in plants of C. quinoa which are also the hosts of the BNYVV virus and which could be more easily manipulated by the man skilled in the art.

The Inventors have also made experiments upon *Beta macrocarpa*. These results have shown that it will be possible to obtain also the transformation of plants by the method according to the invention and obtain expression of TGB3 gene by said plants. Therefore, as explained in the following description, said method could be used to obtain various viral resistances into various plants species subject to infection by viruses characterized by the presence of a TGB3 sequence in their genome.

It is known that BNYVV does not require synthesis of viral coat protein for production of local lesions on leaves of hosts such as *Chenopodium quinoa* (7), indicating that virion formation is not required for cell-to-cell movement.

However, the manner in which the TGB components assist in the movement process is not understood although computer-assisted sequence comparisons have detected characteristic conserved sequences which may provide clues to their function. Thus, the 5'-proximal TGB protein (TGB1) invariably contains a series of sequence motifs characteristic of an ATP/GTP-binding helicase while the second protein (TGB2) always has two potentially membrane-spanning hydrophobic domains separated by a hydrophilic sequence which contains a highly conserved peptide motif of unknown significance (6). The sequence and

size of the third TGB protein (TGB3) is more variable although the N-terminal portion is generally rather hydrophobic. Subgenomic RNAs with 5'-termini mapping upstream of the BNYVV TGB1 and TGB2 open reading frames (ORFs) have been detected (Figure 1) but no such species has been reported for TGB3 of BNYVV (2), or of any other of the TGB-containing viruses. In the case of potato virus X (PVX; ref 8) and barley stripe mosaic virus (BSMV; ref. 9), there is evidence that the TGB2 and TGB3 products are expressed from the same subgenomic RNA.

So far, no example has been reported of a virus in which the three TGB members are arranged differently on the same RNA or are parcelled out to different genome RNAs, suggesting that their association in a particular order might be important in regulating their function.

The present invention concerns a method for inducing viral resistance to a virus comprising a triple gene block (TGB) with the proviso that it is not the potato virus X. Said virus is preferably selected from the group consisting of the apple stem pitting virus, the blueberry scorch virus, the potato virus M, the white clover mosaic virus, the *Cymbidium* mosaic virus; the barley stripe mosaic virus, the potato mop top virus, the peanut clump virus and the beet soil-borne virus; said method comprises the following steps :

- preparing a nucleic acid construct comprising a nucleic acid sequence corresponding to at least 70% of the nucleic acid sequence of TGB3 of said virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,

- transforming a plant cell with the nucleic acid construct, and possibly
- regenerating the transgenic plant from the transformed plant cell.

Preferably, the plant is a plant which may be infected by the above-described virus and is preferably selected from the group consisting of apple, blueberry, potato, clover, orchid, barley, peanut and sugar beet.

The present invention concerns also the obtained plant cell and transgenic (or transformed) plant (made of said plant cells) resistant to said viruses and comprising said nucleic acid construct.

The Inventors have also discovered unexpectedly that it is possible to induce BNYVV-resistance into a plant by a method which comprises the following steps :

- preparing a nucleic acid construct comprising a nucleic acid sequence corresponding to at least 70%, preferably at least 90%, of the nucleic acid sequence of comprised between the nucleotides 3627 and 4025 of the 5' strand of the genomic or subgenomic RNA 2 of the BNYVV or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,
- transforming a plant cell with said construct, and possibly
- regenerating a transgenic plant from the transformed plant cell.

The nucleic acid sequence comprised between the nucleotides 3627 and 4025 of the 5'strand of the genomic or subgenomic RNA 2 encoding the P15 protein is described in the figure 6 and in the publication (3). Said nucleic acid sequence and the corresponding amino acid

sequence are described in the following specifiction as SEQ ID NO. 1.

Another aspect of the present invention concerns a plant cell and a transgenic plant (made of said plant cells) resistant to BNYVV and comprising a nucleic acid construct having a nucleic acid sequence corresponding to at least 70%, preferably at least 90%, to the nucleic acid sequence comprised between the nucleotides 3627 and 4025 of the 5' strand of the genomic or subgenomic RNA 2 of BNYVV or the corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in the plant.

Preferably, said plant cell or transgenic plant (made of said plant cells) resistant to BNYVV is obtained by the method according to the invention.

The variants of the nucleic acid sequence described as SEQ ID NO. 1 comprise insertion, substitution or deletion of nucleotides encoding the same or different amino acid(s). Therefore, the present invention concerns also said variants of the nucleic acid sequence SEQ ID NO. 1 which present more than 70% homology with said nucleic acid sequence and which are preferably able to hybridize to said nucleic acid sequence in stringent or non-stringent conditions.

Preferably, said sequences are also able to induce BNYVV resistance into a plant.

The terms "induce a viral resistance into a plant" mean inducing a possible reduction or a significative delay into the appearance of infection symptoms, virus multiplication or its diffusion mechanisms into the plant, especially in the root tissue.

The regulatory sequence(s) of said nucleic acid sequence are promoter sequence(s) and terminator

sequence(s) active into a plant.

The nucleic acid construct may also include a selectable marker gene, which could be used to identify the transformed cell or plant and express the nucleic acid construct according to the invention.

Preferably, the cell is a stomatal cell and the plant is a sugar beet (*Beta vulgaris ssp.*) made of said cells.

According to the invention, the promoter sequence is a constitutive or foreigner vegetal promoter sequence, preferably selected from the group consisting of 35S Cauliflower Mosaic Virus promoter sequence, polyubiquitin Arabidopsis thaliana promoter (43), a promoter which is mainly active in root tissues such as the par promoter of the haemoglobin gene from Perosponia andersonii (Landsman et al. Mol. Gen. Genet. 214 : 68-73 (1988)) or a mixture thereof.

A last aspect of the present invention is related to a transgenic plant tissue such as fruit, stem, root, tuber, seed of the transgenic plant according to the invention or a reproducible structure (preferably selected from the group consisting of calluses, buds or embryos) obtained from the transgenic plant or the cell according to the invention.

The techniques of plant transformation, tissue culture and regeneration used in the method according to the invention are the ones well known by the person skilled in the art. Such techniques are preferably the ones described in the International Patent Applications WO95/10178 or WO91/13159 corresponding to the European Patent Application EP-B-0517833, which are incorporated herein by reference. These techniques are preferably used

for the preparation of transgenic sugar beets according to the invention.

Brief description of the drawings

The figure 1 represents Structure of wild-type BNYVV RNA 2 and of replicons expressing TGB proteins.

The figure 2 represents the *In vitro* translation of the replicons of the TGB genes in wheat germ extract.

The figure 3 represents the amplification of the replicons encoding the TGB proteins in *Chenopodium quinoa* protoplasts and expression of P42.

The figure 4 represents the complementation of RNA 2 transcripts containing defects in the different TGB genes by the corresponding wild-type gene supplied from a replicon.

The figure 5 shows the effect of replicons on infection with wild-type BNYVV RNAs 1 and 2.

The figure 6 represents the nucleotides and amino acid sequence of TGB3 encoding the P15 of BNYVV.

The figure 7 shows the presence of the coding regions of the BNYVV p15 gene in the sugar beet genome by a PCR.

The figure 8 shows the integration of the BNYVV P15 gene in the sugar beet genome by Southern blot hybridisation.

Description of the invention

In order to identify the potential use of particular gene sequences isolated from the viral RNA2 of BNYVV and to create BNYVV-resistance in a sugar beet via plant transformation, the inventors have investigated

whether independent expressions of the BNYVV TGB protein is possible by inserting the ORF of each into a viral replication-dependant "replicon" derived from BNYVV RNA3. The Inventors have showed that in mixed infections of *C. quinoa* leaves, the BNYVV TGB1 or TGB2 protein so expressed can complement BNYVV RNA 2 containing a mutation disabling the corresponding TGB protein. No complementation was observed with a replicon containing TGB3, however, unless the TGB3 ORF was positioned downstream of the ORF for TGB2 in the replicon. When co-inoculated with wild-type RNAs 1 and 2, the replicon expressing the TGB3 ORF of BNYVV inhibited infection. The data are consistent with a model for expression of the TGB proteins in which translation of P15 from a dicistronic subgenomic RNA regulates P15 expression levels *in vivo*. The Inventors have also identified that high expression of P15 could ensure a rapid and total blocking of virus multiplication and diffusion mechanisms in the plant.

MATERIALS AND METHODS

cDNA clones

The transcription vector for production of wild-type full-length BNYVV RNA 1 and RNA 2 were pB15 (10) and pB2-14 (11), respectively. Transcription vectors for previously described RNA 2 mutants were pB2-14-F, -H, -I and -J (2) and pB2-14-ΔSN, -ΔS12, -ΔS37, -ΔB1, -ΔB2, -ΔB2, -ΔN and -GAA (11). The RNA 2 deletion mutant pB2-14- HP1 was produced by elimination of the sequence between nucleotides 3158 and 3258. The empty BNYVV RNA 3-derived replicon, rep0, was obtained by transcription of the RNA 3 deletion mutant pB35AΔES (12). TGB sequences for insertion into rep0 were amplified by the polymerase chain reaction

(PCR) using primers which each contained a non-templated *Bam*HI site at their 5'-extremity. PCR fragments corresponding to the P42 gene (nucleotides 2127-3297), the P13 gene (nucleotides 3282-3650), the P15 gene (nucleotides 3627-4025) and both the P13 and P15 genes (nucleotides 3282-4025) were digested with *Bam*HI and inserted into *Bam*HI-cleaved pB35AΔES. The resulting constructs were used to transcribe rep42, rep13, rep15, and rep1315, respectively. A replicon containing a frameshift mutation in the P15 ORF (Rep15-X) was produced by filling in the overhangs of an insert *Xba*I site (nucleotide 3948). The insert frameshift mutations in rep13-I, rep1315-I and rep15-J were created as described for the corresponding mutations in full-length RNA 2 (2). Cloned PCR-amplified sequences were verified to be error-free by sequencing (13).

In vitro transcripts

Capped transcripts were prepared by bacteriophage T7 polymerase run-off transcription (10) of plasmid DNA linearized by *Hin*dIII for pB15 and the replicon constructs and by *Sal*I for pB2-14 and related constructs. Transcript concentration and integrity were evaluated by agarose gel electrophoresis. Leaves were mechanically inoculated with 50 µl per leaf of inoculation buffer containing 1 µg of each transcript (2). In some experiments, the RNA 1 and 2 transcripts were replaced by 0.025 µg of the highly infectious viral RNA purified from BNYVV isolate Stras 12 (10). Preliminary experiments showed that this amount of viral RNA was approximately equivalent in infectivity as measured by a local lesion assay to a mixture containing 1 µg each of the RNA 1 and 2

transcripts. For protoplast infections, 0.5 µg of viral RNAs 1 and 2 plus 3 µg replicon transcript were inoculated to 2.10^5 protoplasts by electroporation (2).

Transcripts obtained from replicons were translated in a wheat germ extract (14) and the [^{35}S]-labelled translation products were visualized by autoradiography after SDS-PAGE (15, 16). Radioactivity incorporated into translation products was quantified with a Fujix MAS1000 BioAnalyzer and the values were adjusted for methionine content in calculating relative translation levels.

Detection of viral RNA and proteins

Total RNA was extracted (2) from inoculated leaves 10 days post-inoculation (pi) and from protoplasts 48 hr pi. Viral RNA was detected by northern hybridization with ^{32}P-labelled antisense viral RNA transcripts (17) as probes. The RNA 1-specific probe was complementary to nucleotides 4740-5650, the RNA 2-specific probe to nucleotides 2324-3789 and the RNA 3-specific probe to nucleotides 1-380. P42, P14 and coat protein were detected by Western blot of total protein extracts of infected protoplasts using a rabbit polyclonal antiserum specific for each protein (18). The stability of mutations introduced into RNA 2 was tested by the polynucleotide chain reaction following reverse transcription (RT-PCR) of total RNA extracts from infected plants. Reverse transcripts were produced with an Expand™ reverse transcription kit (Boehringer) following the manufacturer's instructions. PCR was carried out essentially as described (19) using 25 cycles of the following regimen: 94 °C (30

sec), 50 °C (30 sec), 72 °C (3 min). Primer pairs for PCR amplification of different regions of the RNA 2 cDNA corresponded to (or were complementary to, in the case of the second member of each pair of primers) nucleotides 1143-1151 and 3393-3412 (P42 gene), and nucleotides 3151-3169 and 4128-4148 (P13 and P15 genes). The primer used to initiate cDNA synthesis prior to the PCR reactions was complementary to nucleotides 4128-4148.

RESULTS

Replicons expressing the BNYVV TGB proteins

Provided that sufficient sequences at the 3'- and 5'-extremities are retained, a BNYVV RNA 3 transcript from which the central region has been deleted can replicate efficiently on *C. quinoa* leaves when coinoculated with RNAs 1 and 2, and can express a foreign gene inserted in place of the deleted sequence (12, 20). The Inventors have used such an RNA 3-based "replicon" to express each of the BNYVV TGB proteins out of their normal context in RNA 2 and tested the capacity of each replicon to complement an RNA 2 mutant defective in the corresponding TGB gene.

The replicons employed in this study are depicted in Figure 1. Figure 1 A is the Genome map of RNA 2. The TGB genes are shaded and lines above the map indicate the extents of the subgenomic RNAs $2sub_a$ and $2sub_b$. The positions of deletions and frameshift-inducing insertions in RNA 2 are indicated. The 5'-terminal cap structure is denoted by a circle. P21 is the major viral coat protein. RT = readthrough domain (3 . (B) BNYVV RNA 3-derived replicons containing the TGB genes of BNYVV (light shading) or the TGB3 gene encoding P1? of peanut clump

virus (PCV) (dark shading). The *Bam*HI site in the empty replicon (rep0) used for insertion of the PCR-amplified TGB sequences is shown. The positions of frameshift-inducing insertions in the various P13 and P15 mutant replicons are indicated. In addition to the constructs rep42, rep13 and rep15, which each contain a TGB gene, a fourth construct (rep1315) was produced containing both the P13 and P15 genes arranged in the same relative configuration as in RNA 2. The ability of each replicon to direct expression of the inserted gene or genes was tested by *in vitro* translation of the transcript in a wheat germ extract. The rep42, rep13 and rep15 transcripts each directed synthesis of an abundant product (Figure 2A, lane 2; Figure 2B, lanes 2 and 3), which was not produced in translations programmed with transcript corresponding to the empty replicon, rep0 (Figure 2A, lane 1; Figure 2B, lane 1). In the figure 2 (A), are represented S^{35}-methionine-labelled translation products of the empty replicon rep0 (lane 1) and rep42 (lane 2) displayed by autoradiography following PAGE (15). The indicated band was identified as P42 by comparison of its mobility to that of molecular weight markers (not shown). In the figure 2 (B), are represented translation products directed by rep0 (lane 1), rep13 (lane 2), rep15 (lane 3) and rep1315 (lane 4) displayed by autoradiography following PAGE (16). The bands tentatively identified as P13 and P15 are indicated to the right. The background band denoted by an asterisk was also synthesized when no transcript was introduced into the translation extract. The relative mobilities of the various translation products were as expected except that the putative P13 migrated slightly more slowly than P15, presumably because of its nontypical amino acid composition. The dicistronic

construct rep1315 directed synthesis of both P13 and P15 (Figure 2B, lane 4) in relative molar amounts of 3:1 (values corrected for the difference in methionine contents of the two proteins; if the N-terminal methionine of each protein is removed post-translationally, the molar ratio is 5:1).

The capacity of the replicons to be amplified by the viral replication machinery *in vivo* was tested by coinoculating replicon transcripts to *C. quinoa* protoplasts along with BNYVV RNA's 1 and 2. Northern blot analysis of total RNA extracted from the protoplasts 48 hr pi revealed that all the replicons containing the TGB genes were efficiently amplified (Figure 3A). The figure 3 (A) represents detection by northern hybridization of viral RNAs in *C. quinoa* protoplasts inoculated with BNYVV RNAs 1 and 2 alone (lane 2) or supplemented with rep0 (lane 3), rep42 (lane 4) rep13 (lane 5) rep1315 (lane 6) and rep15 (lane 7). RNA from mock-inoculated protoplasts was analyzed in lane 1. The protoplasts were harvested 48 hr pi and viral RNAs were detected using ^{32}P-labelled viral RNA-specific antisense RNA probes. The replicons are indicated by arrow heads. The figure 3 (B) represents Immunodetection of P42 in total protein extracts of *C. quinoa* protoplasts inoculated with BNYVV RNAs 1 and 2 (lane 2), transcript of wild-type RNA 1 plus transcript of the RNA 2 mutant pB2-14-H, which contains a frameshift mutation in the P42 gene (11) (lane 3), the RNA 1 and pB2-14-H transcripts plus rep42 (lane 4). Protein extracted from mock-inoculated protoplasts was analyzed in lane 1. After PAGE (15) and electrotransfer to nitrocellulose, P42, major viral coat protein (CP) and P14 were immunodetected with a mixture of antisera specific for each protein (18). The positions of

molecular weight standards are labelled in kilodaltons to the left of the blot. Western blot analysis revealed that the P42 level in protoplasts infected with a mixture of rep42 plus transcripts of RNA 1 and the frameshift mutant pB2-14-H, caused by filling in an *Spe*I site within the P42 gene of RNA 2 (see Figure 1), was about twice that in protoplasts infected with RNA 1 plus wild-type RNA 2 (Figure 3B, lanes 2 and 3). Note that the levels of accumulation of two other immunodetectable RNA 2 gene products (the major viral coat protein and P14; Figure1) were not modified by the presence of rep42. P13 and P15 could not be immuno-detected in such experiments.

The BNYVV TGB proteins can be complemented *in trans*

The ability of the replicons containing the TGB genes to supply movement functions in whole plants was tested by coinoculating leaves of the local lesion host *C. quinoa* with one of a series of RNA 2 transcripts containing a mutation disabling a TGB gene plus a replicon containing the corresponding wild-type gene. In all experiments, the inoculum also contained transcript of wild-type RNA 1 as source of viral RNA-dependent RNA replicase, although this fact will not always be stated explicitly below. For the P42 gene, the RNA 2 mutants tested included the frameshift mutant (pB2-14-H) caused by filling in an *Spe*I site at nucleotide 2280, a series of mutants containing short in-frame deletions at different positions in the P42 ORF (mutants pB2-14-ΔS12, -ΔSN, -ΔB1, -ΔB2, -ΔN, and -ΔHP1 ; Figure 1A; also see ref. 11), and a deletion mutant (pB2-14-F; Figure 1) where removal of a 935 nucleotide sequence upstream of the P42 ORF has inactivated the promoter for the subgenomic RNA (RNA $2sub_a$) responsible for P42

synthesis. Inocula containing RNA 1 transcript plus any one of the above mutant RNA 2 transcripts did not produce local lesions on *C. quinoa* and no progeny viral RNA could be detected in the inoculated leaves 10 days pi (Figure 4, lanes 3 and 5; see ref. 11 for the other mutants). In the figure 4, the replicon indicated at the top of each lane was inoculated to leaves of *C. quinoa* together with wild-type RNA 1 transcript plus either wild-type RNA 2 transcript (lane 2) or the mutant RNA 2 transcript identified above each lane. In lanes 19 and 20 the inoculum contained rep42 and rep15 (lane 19) or rep42 and rep1315 (lane 20), in addition to RNA 1 and pB2-14- HP1 transcripts. Lane 1 contains RNA from a non-inoculated control plant. Inoculated leaves were harvested 10 days pi and tested for viral RNA contents by northern hybridization as described in Figure 3. The positions of replicons are indicated by arrows. When rep42 transcript was included in the inoculum, numerous local lesions (20-80 per leaf) appeared on the inoculated leaves except for the inoculum containing transcript of the RNA 2 deletion mutant pB2-14-HP1, which remained symptomless . The resulting pale green lesions were similar in appearance to those elicited by inoculation with RNA 1 plus wild-type RNA 2 except for the RNA 2 mutant pB2-14-F, where necrotic local lesions were formed. In this later case, the necrotic lesion phenotype may be related to production of a truncated form of the readthrough (RT) protein by this RNA 2 mutant (7).

Northern hybridization of the inoculated leaves 10 days pi revealed the presence of progeny viral RNAs of the length expected for RNAs 1, 2 and rep42 for all the RNA 2 mutants (Figure 4, lanes 2, 4, 7-11 except the deletion mutant pB2-14- HP1 Figure 4 lane 13). As will be

shown below, the failure of pB2-14-ΔHP1 to be complemented by rep42 is probably due to deletion of the promoter for the subgenomic RNA (RNA 2sub$_b$), which is believed to direct translation of the downstream TGB proteins.

Similar complementation experiments were carried out with rep13, rep15 and the dicistronic construct rep1315. Both rep13 and rep1315 were able to complement accumulation on leaves (Fig 4, lanes 14 and 15) of the mutant pB2-14-I, in which the P13 gene had been disabled by insertion of four nucleotides (the insertion created an *Xho*I site), although the resulting local lesions were necrotic. Necrotic local lesions were also produced during mixed infections with the aforesaid replicons and wild-type RNA 1 and 2 (see below), indicating that the replicon-related symptom phenotype is dominant over the wild-type. The novel symptoms may be related to differences in the time course of synthesis or the level of accumulation of P13 when it is expressed from the replicon rather than full-length RNA 2.

In experiments such as those described above, it is important to demonstrate that the mutation originally introduced into the P42 or P13 gene on the RNA 2 transcript was still present in the progeny RNA 2, that is, the defective copy of the TGB gene on the transcript had not been converted to the wild-type by RNA recombination *in planta* (21) with the copy present on the replicon. Therefore, an RT-PCR experiment was carried out on the progeny viral RNA from a plant infected with RNA 1, pB2-14-H transcript (P42 gene disrupted by filling in an *Spe*I site) and rep42. The primer pair used in the RT-PCR hybridized to RNA 2 sequences flanking the P42 gene and hence amplifies the copy of the gene present in RNA 2 but

not the copy on the replicon, where the flanking sequences are absent. Restriction enzyme analysis revealed that the *SpeI* site was absent in the resulting amplified DNA fragment, as expected for the mutated rather than the wild-type form of the TGB gene. Similar analysis of progeny viral RNA from plants infected with RNA 1, pB2-14-I (frameshift mutation in P13 gene creating an*Xho*I site) and either rep13 or rep1315 similarly demonstrated that the mutation disabling the copy of the P13 gene on the RNA 2 transcript was conserved in the progeny RNA 2. We conclude that rep42 and rep13 are indeed complementing P42 and P13 function by supplying the gene product *in trans* rather than simply serving as a source of the wild-type TGB sequence for recombination.

Unexpectedly, the replicon expressing the wild-type P15 gene (rep15) was unable to complement the P15-defective RNA 2 mutant pB2-14-J in mixed inoculations. No local lesions formed on the inoculated leaves 10 days pi and no viral RNA could be detected in the leaves by northern blot (Fig 4, lane 17). On the other hand, when pB2-14-J transcript was coinoculated with rep1315, local lesions (of the necrotic type) appeared and progeny viral RNAs were readily detected (Figure 4, lane 18). In this latter case, analysis of an RT-PCR product containing the P15 gene in the progeny RNA 2 revealed that the mutation disabling the gene was still present. Complementation of pB2-14-J still occurred when the P13 ORF in the dicistronic replicon was interrupted by a frameshift mutation (rep1315-I; Figure 1), establishing that expression of full-length P13 from the first ORF of the dicistronic replicon is not required for complementation by the downstream copy of the P15 gene.

Evidence that P15 is expressed from a dicistronic subgenomic RNA

An RNA 2-derived subgenomic RNA (RNA $2sub_b$) of about 1500 nucleotides length has been detected in BNYVV-infected tissue (2). The 5'-extremity of this species has not been mapped precisely but is predicted to lie near the 5'-terminus of the P13 ORF. No subgenomic RNA with 5'-end upstream of the P15 ORF has been detected, raising the possibility that, as in BSMV (8), P13 and P15 are both expressed from RNA $2sub_b$.

The aforementioned inability of rep42 to complement the P42-defective RNA 2 mutant pB2-14- HP1 could stem from polar effects of the RNA 2 deletion on synthesis of downstream TGB proteins if the deletion has disabled the RNA $2sub_b$ promoter (The right-hand boundary of the deletion in pB2-14-ΔHP1 is only 30 residues upstream of the P13 initiation codon). To test this hypothesis, an experiment was carried out in which the pB2-14-ΔHP1 transcript was complemented with both rep42 and rep1315. Leaves inoculated with this mixture developed local lesions and contained progeny viral RNA's (Figure 4, lane 20). If, on the other hand, rep13 rather than rep1315 was used along with rep42 to complement pB2-24- HP1, no symptoms appeared and no progeny viral RNA's were detected by northern blot (Figure 4, lane 19). These observations are consistent with the hypothesis that the pB2-14- HP1 deletion interferes with expression of the downstream TGB ORF's, presumably by blocking RNA $2sub_b$ transcription. Furthermore, the fact that complementation was successful with rep1315 but not with rep13 indicates that P15 as well as P13 is translated from RNA $2sub_b$.

Independent expression of P15 inhibits infection with wild-type viral RNA

The ability of rep1315 but not rep15 to complement the P15-defective RNA 2 mutant pB2-14-J in leaf infections could indicate that independent expression of P15 from the monocistronic replicon interferes with the viral infection cycle by producing the gene product in excessive quantities relative to P13. To test this hypothesis, an experiment was carried out in which rep15 was inoculated to *C. Quinoa* leaves along with wild-type viral RNAs 1 and 2. No lesions appeared on the inoculated leaves, even at late times pi (Figure 5A), and no viral RNA could be detected by northern blot (Figure 5B, lane 6). The figure 5 (A) represents leaves of *C. quinoa* inoculated with RNAs 1 and 2 (left) or RNAs 1 and 2 plus rep15 (right). The leaves were photographed 20 days pi when the local lesions on the leaf to the left had expanded so as to cover much of the leaf surface. In the figure 5 (B), analysis by northern hybridization (as described in Figure 3) of the viral RNA contents of *C. quinoa* leaves inoculated with BNYVV RNAs 1 and 2 alone (lane 1) or together with rep0 (lane 2), rep42 (lane 3), rep13 (lane 4), rep1315 (lane 5), rep15 (lane 6), rep15-J (lane 7), rep15-X (lane 8) or repPCV-P17 (lane 9). The positions of replicons are indicated by arrows. (C) Analysis by northern hybridization of the viral RNA contents of the inoculated leaves (lanes 1, 3 and 5) and the roots (lanes 2, 4 and 6) of *Beta macrocarpa* either mock-inoculated (lanes 1 and 2), inoculated with BNYVV RNAs 1, 2 and 3 (lanes 3 and 4) or with RNAs 1, 2 and 3 plus rep15 (lanes 5 and 6). RNA 3 was included in the inoculum because it is necessary for systemic movement in *B. macrocarpa* (22). Under these conditions, leaves inoculated

with RNAs 1 and 2 alone were heavily infected (Figure 5A; Figure 5B, lane 2). The inhibition of virus infection by rep15 was dose-dependent. Addition of ten times less rep15 to the inoculum mix still resulted in almost complete inhibition of lesion formation but lesser amounts of the replicon were progressively less effective in blocking the infection. Rep15 also blocked the appearance of progeny viral RNA in the inoculated leaves and in the roots of *Beta macrocarpa*, a systemic host of BNYVV (Figure 5C, lanes 5 and 6). The empty replicon, rep0, and replicons expressing the other two TGB proteins (rep42, rep13, rep1315), on the other hand, did not significantly inhibit BNYVV infection of *C. quinoa* leaves (Figure 5B, lanes 2-5).

Since rep15 did not interfere with amplification of RNA 1 and 2 in *C. quinoa* protoplasts (see Figure 3), this suggests that the replicon interferes with movement of the virus from the initial site of infection into neighbouring cells (cell-to-cell movement) during local lesion formation on leaves. Lesion formation was not inhibited by coinoculation of Stras 12 RNA with the replicons rep15-J or rep15-X (Figure 5B, lanes 7 and 8), which encode frameshift-truncated forms of P15. This finding confirms that expression of P15 from the replicon, rather than the simple presence of the corresponding RNA sequence, is required for inhibition during the mixed infection experiments. In the presence of rep15-X, however, the resulting local lesions were about one third the diameter of the lesions formed by infection with Stras 12 alone or with Stras 12 plus rep15-J and the content of progeny viral RNA in the infected leaves was significantly lower (Figure 5B, lane 8). This finding suggests that the almost full-length P15 molecule produced by rep15-X,

although incapable of substituting for wild-type P15 in a complementation experiment, can interfere with the cell-to-cell movement activity of the wild-type P15 produced from RNA 2. Presumably, the full-length and truncated forms of P15 compete with one another for binding sites on another component (which could be of either viral or cellular origin) involved in the movement process.

As noted above, sequence comparisons between different viruses possessing a TGB have revealed little sequence similarity between the different TGB3 genes. For example, the 17 kDa TGB3 protein (P17) of peanut clump furovirus (PCV) displays no significant sequence similarity with P15 of BNYVV (4) even though both viruses can infect *C. quinoa*. To determine whether independent expression of the PCV TGB3 protein can interfere with a BNYVV infection in a manner similar to that observed with rep15, a BNYVV RNA 3-derived replicon containing the PCV TGB3 (repPCV-P17; Figure 1) was constructed. *C. quinoa* leaves inoculated with BNYVV RNAs 1 and 2 plus repPCV-P17 did not develop symptoms and no progeny BNYVV RNA's could be detected by northern blot (Figure 5A, lane 9). This observation suggests that the pathways by which BNYVV and PCV move from cell-to-cell in *C. quinoa* share at least one common element which, in spite of their dissimilarity in sequence, interacts with the TGB3 products of both viruses.

The Inventors have shown that replicons carrying P42 and P13 can complement BNYVV RNA 2 carrying the corresponding defective gene but that a replicon carrying P15 cannot. In the latter case, complementation can occur, however, if the P15 gene is supplied as the second gene on a dicistronic RNA (rep1315) carrying the P13 gene in first position. It should be noted that the

relative disposition of the P13 and P15 genes on rep1315 is identical to their disposition on RNA 2sub$_b$, the subgenomic RNA which is believed to direct synthesis of both proteins in wild-type infections. This suggests that successful cell-to-cell movement of BNYVV requires the presence of P13 and P15 in appropriate relative amounts and that production of both proteins from the same subgenomic RNA represents a mechanism for coordinating their synthesis. The inability of rep15 to complement the P15 mutant RNA 2 transcript pB2-14-J and its ability to inhibit infection by wild-type virus would then both be due to over-production of P15 relative to P13 when the former is translated from the replicon and the latter from RNA 2. When P15 is expressed from the dicistronic replicon rep1315, on the other hand, appropriate relative P13-P15 levels would be produced, allowing cell-to-cell movement to proceed. The "correct" relative levels of accumulation of P13 and P15 in a wild-type infection are not known. Translation of rep1315 in wheat germ extract produced three to five times more P13 than P15 but such experiments do not necessarily reflect the situation *in planta* since the turnover rates of the two proteins may differ significantly. Note that these results indicate that TGB-mediated cell-to-cell movement is less sensitive to over-expression of P42 and P13 relative to the "correct" levels characteristic of a normal infection since co-inoculation of rep42, rep13 or rep1315 with wild-type virus did not inhibit infection (Figure 5, lanes 3-5), although the lesions produced in the presence of rep13 and rep1315 were necrotic. Thus, it shows that expression of P15 in transgenic plants could provide a mechanism for inducing BNYVV-resistance ("pathogen-derived resistance"; ref. 23) in such plants, provided that sufficient P15

expression levels can be attained.

To gain a better understanding of how the relative levels of P13 and P15 are regulated during translation will require learning how the P15 cistron is accessed by ribosomes on RNA 2sub$_b$. Translation initiation at an internal cistron of a eucaryotic messenger RNA may occur by several mechanisms, including (i) leaky scanning, where a fraction of the ribosomal subunits which begin scanning the RNA at the 5'-end move past the first (non-optimal) upstream AUG without initiating (24), and (ii) internal entry, where ribosomal subunits bind directly to a special sequence on the RNA near the internal initiation codon (25). A third possible mechanism, termination-reinitiation (24), appears unlikely to apply to any of the TGB-containing viruses because the overlap between the TGB2 and TGB3 cistrons would require ribosomes to scan backwards after terminating TGB2 to reach the TGB3 initiation codon. It has been suggested that the TGB3 proteins of BSMV and PVX are translated by a leaky scanning mechanism (8, 9). The BNYVV P15 gene may also be produced by leaky scanning although it should be noted, however, that the context of the BNYVV P13 initiation codon (AUAAUGU) is nearly optimal and there are also two downstream AUG's which scanning subunits would have to ignore to reach the P15 initiation codon.

EXAMPLES

The following examples are transformation of plant made by the technique described in the International Patent Application WO95/[illegible] incorporated herein by reference.

The plant material and growth conditions were the ones described by Hall et al., Plant Cell Reports 12, pp. 339-342 (1993) Pedersen et al., Plant Science 95, pp. 89-97 (1993), and Hall et al, Nature biotechnology 14, 1996, in press.

Plasmid vectors and DNA preparation

The plasmid pET-P15 (harbouring the P15 nucleic acid sequence) was restricted at its single BamHI site and blunt-ended with T4 DNA polymerase. After purification by electrophoresis in 0.8% agarose gel, the linear plasmid was restricted at its single NcoI site. The P15 gene fragment of 400 bp was purified by electrophoresis and inserted into pMJBX-Ub (harbouring the Arabidopsis polyubiquitin promoter (Norris et al., Plant Molecular Biology 21, pp. 895-906 (1993), a TMV enhancer sequence and the Nos 3' terminator) cut with NcoI and SmaI restriction endonucleases. In the plasmid so obtained (pMJBX-Ub-P15), the nucleic acid sequence of the P15 gene is placed under the control of the Arabidopsis polyubiquitin promoter followed by the TMV enhancer sequence. The EcoRI fragment from plasmid pB235SAck contains the pat gene, used as the selective marker, encoding phosphinothricin acetyl transferase (obtained from Agrevo, Berlin Germany). On this EcoRI fragment, the nucleic acid sequence of the pat gene is under the control of the 5' and 3' expression signals of the Cauliflower virus. The plasmid pMJBS6, resulting from the combination of this EcoRI-pat fragment and a partial EcoRI digestion of plasmid pMJBX-Ub-P15, contains both the pat and the P15 genes. This pMJBS6 plasmid is a high-copy plasmid based on the pUC18 vector and contains also the -lactamase gene

(amp^r). In the plasmid pIGPD7, harbouring the same pat fragment as pB235SAck, the -lactamase gene was replaced by an igpd (imidazole glycerol phospnate dehydratase) gene from Saccharomyces cerevisiae (Struhl et al., Proceedings of the National Academy of Science USA 73, pp. 1471-1475 (1976). Selection for and maintenance of the plasmid in Escherichia coli was achieved by complementation of an auxotrophic hisB strain SB3930 on minimal medium in the absence of antibiotics. The P15 fragment, with its ubiquitin promoter and terminator sequence, was purified as a 2500 bp fragment obtained from the pMJBX-Ub-P15 plasmid after it was cut at the single HindIII site, followed by a partial EcoRI restriction. This fragment was blunt-ended and inserted in a blunt-ended pIGPD7 plasmid, cut at the single NcoI site. The resulting pIGPDS4 plasmid contains both the pat and the P15 genes on a vector without the ß-lactamase gene.

Plant material

In vitro shoot cultures of sugar beet plantlets were initiated to provide a reusable and uniform source of sterile starting material and were maintained with a 4-weekly subculture period as described by Hall et al., Plant Cell Reports 12, pp. 339-342 (1993).

Large-scale isolation of sugar beet epidermis

A modified version of the blender method by Kruse et al., Plant Physiology 690, pp. 1382-1386 (1989) was used. For each isolation, 2 g leaves (with the midribs removed) from 4 week old shoots was blended in a Waring blender at maximum speed (23000 rpm) for 60 sec in a 250 ml

metal beaker containing 50 ml cold (4 °C) Ficoll medium (100 g /l Ficoll, 735 mg/l $CaCl_2.2H_2O$, 1 g/l PVP40, autoclaved). The epidermal fragments were then recovered on a 297 µm nylon filter and washed with 500 ml sterile tap water. These were rinsed from the filter into a 9 cm Petri dish using 10 ml CPW9M containing 3.8% (w/v) $CaCl_2.2H_2O$ (Krens et al., Theoretical and Applied Genetics 79, pp. 390-396 (1990). Any remaining leaf fragments were removed and dishes were preincubated for 1 h at room temperature.

Guard cell protoplast isolation from enriched epidermis fractions

To recover the epidermis fraction, the suspension was centrifuged for 1 min at 55 x g after which the supernatant was removed. The pellet was resuspended in 50 ml enzyme mix and 5 ml aliquots were transferred to each of 10, 6 cm Petri dishes (Greiner, TC quality), sealed with parafilm and incubated overnight at 25 ° C in darkness with gentle agitation. The digestion medium consisted of CPW9M supplemented with 0.5% (w/v) Cellulase RS and 3% (w/v) Macerozyme R10 (Yakult Honsha, Tokyo, Japan), pH 5.8. The following morning, the protoplasts were generally seen floating near the surface of the digestion mix. After gentle agitation of the suspensions using a sterile pipette to release the protoplasts still adhering to cuticle fragments, the digestions were pooled and passed through 297 and 55 µm nylon filters. The filtrate was mixed with an equal volume of iso-osmotic Percoll containing 15% (w/v) sucrose (Percoll15S) and divided over 12 x 12 ml centrifuge tubes. In each tube, first 1 ml CPW15S (Krens et al., 1990) and then 0.5 ml 9% (w/v) mannitol containing 1 mM $CaCl_2$

(9M) were carefully layered on top of the protoplast suspension. After centrifugation at 55 x g for 10 min the viable guard cells were visible in bands at the CPW15S/9M interface. To concentrate the protoplasts, these bands were collected and mixed with Percoll15S to give a final volume of 16 ml. This was then divided between 2 centrifuge tubes, layered as above and recentrifuged. Careful removal of the 9M layers yielded the enriched guard cell protoplast fraction for subsequent counting using a haemocytometer.

Protoplast transformation

Transformations were performed in 12 ml centrifuge tubes, each containing 1 x 10^6 protoplasts suspended in 0.75 ml 9M medium. Plasmid DNA (50 μg of pMJBX-Ub-P15 and pIGPDS4) was added and, immediately after mixing, 0.75 ml PEG medium was added dropwise (40% PEG 6000 dissolved in F medium (Krens et al., Nature 296, pp. 72-74, (1982). After thorough mixing, the suspension was kept at room temperature for 30 min with intermittent agitation. Subsequently, at 5 min intervals, 4 x 2 ml aliquots of F medium were added. After centrifugation for 5 min at 55 x g the supernatant was discarded and the protoplast pellet resuspended in 9M and recentrifuged. The cells were finally resuspended in 1 ml of 9M medium for counting.

Protoplast culture and selection

Protoplasts were embedded in Ca alginate and cultured in modified, liquid K8P medium (Hall et al., 1990). To select for stably transformed cells, bialaphos, the active compound of Herbiace (Meiji Seika Ltd, Japan) was added after 7 days, to give a final concentration of 200 µg/l. On day 14, the alginate discs were cut into 3 mm

slices and transferred to PGo medium (De Greef and Jacobs, Plant Science Letters 17, pp. 55-61, 1979) supplemented with 1 μM BAP (PG1B) and 250 μg/l bialaphos and solidified with 0.8% agarose.

Callus culture and regeneration

After 21 days, the pieces of alginate containing the non-visible microcalli were transferred to 9 cm Petri dishes containing 20 ml Medium K (3% sucrose, 0.8% agarose, 1 μM BAP, PGo medium, pH = 5.8 autoclaved). Culture was in the dark as above.

Friable, watery-type calli on reaching the size of approximately 1-2 mm in diameter, were individually picked off and cultured in groups of 20 on fresh Medium K. At this stage, PCR analyses confirmed the presence of transformants.

At two-weekly intervals all calli were subcultured on to fresh medium.

Regenerants appeared during the first 8 weeks of culture of individual calli. When the first shoots were visible and had reached a size of approximately 2 mm, the dish was transferred into the light (3000 lux), 25 ^{o}C, 15 hour day length.

Plantlets approximately 4 mm long were transferred to individual culture tubes containing 15 ml of Medium K and were further subcultured in the light as above.

Rooting and transfer to the soil

When the plantlets had reached the four-leaf stages (usually after 5 to 6 weeks with one subculture

after 3 weeks), they were transferred to culture tubes containing 15 ml of Medium L (3% sucrose, 0.8% agarose, 25 µM indolebutyric acid (IBA), PGo medium, pH = 5.8 autoclaved) (PGo medium described by De Greef W. et al., Plants Science Letters 17, pp. 55-61 (1979)) and further cultured as above.

When at least one root had reached a length of 1 cm, the plantlets were removed from the culture tubes and washed under running tap water to remove all fragments of the agar, and transferred to soil in 9 cm pots in the greenhouse.

Plantlets were covered with a transparent plastic cup to provide a humid environment for 7 days, after which they could be grown without protection.

The plant transformed by the sequence SEQ ID NO. 1 according to the invention is recovered and has been expressed P15.

DNA analysis

Genomic DNA isolated from the primary transformants is electrophoresed in a 0.8% agarose gel after treatment with restriction enzymes and transferred to a nitrocellulose membrane using standard procedures, according to the manufacterer's protocol. Hybridisation is performed with the DNA, as α^{32}P-dATP-labelled probes, whose presence it is desired to establish. The membranes were washed to a final stringency of 0.1% x SSC, 0.1% SDS at 60 °C. The hybridized DNA is visualised by darkening the X-ray film for 24 to 48 hours.

PCR analysis

Standard PCR techniques were used to detect a range of intact plasmid sequences. Reactions were performed using 25 cycles of 1 min denaturation at 94 °C, 1 min annealing; 2 min extension at 72 °C, with a final extension period of 5 min. The annealing temperatures were optimized for each primer combination. The presence of the coding region of the BNYVV P15 gene in the sugarbeet genome was verified by PCR using a pair of oligonucleotides as primers : MOV1, sense primer [5'-GGTGCTTGTGGTTAAAGTAGATTTATC-3' (nucleotides 3 to 29 on SEQ ID NO 1)] and MOV2 antisense primer [5'-CTATGATACCAAAACCAAACTATAGAC-3' (complementary to the nucleotides 369 to 395 on SEQ ID NO 1)]. This 393 bp long fragment comprises the whole coding region of the P15 gene for BNYVV (see figure 7).

Figure 7: Analysis of PCR products obtained with sugarbeet DNA from P15-transformants (lane 5 to 7) and an untransformed plant (lane 4). Low DNA Mass Ladder® (Life Technologies) was used as a size marker (lane 1). Lane 2 and 3 correspond to the positive controls (pMJBS6/pIGPDS4). The arrow on the left shows the position of the expected PCR product.

Southern blot hybridisation analysis

The integration of the BNYVV P15 gene in the sugarbeet genome was verified by Southern blot hybridisation. Total DNA of primary transgenic regenerants was isolated, digested with restriction enzymes (PstI, KpnI, NcoI, SacI), electrophoresed, blotted and hybridised with BNYVV P15 specific ^{32}P-labelled probes using PCR amplified MOV1-MOV2 fragment (see figure 8).

Figure 8: Southern blot analysis. Lambda DNA digested with HindIII was used as a size marker (lane 1). Lanes 2 to 16, DNA of the transgenics: 2 to 4 digested with SaCI, 6 to 8 digested with PstI, 10 to 12 digested with NcoI, 14 to 16 digested with KpnI. Lanes 5, 9, 13 and 17 correspond to the untransformed plant.

REFERENCES

1. Richards K.E. & Tamada T., *Annu. Rev. Phytopathol.* **30**, pp. 291-313 (1992)
2. Gilmer D. et al., *Virology* **189**, pp. 40-47 (1992)
3. Bouzoubaa S. et al., *J. Gen. Virol.* **68**, pp. 615-626 (1987)
4. Herzog E. et al., *J. Gen. Virol.* **18**, pp. 3147-3155 (1994)
5. Scott K. P. et al., *J. Gen. Virol.* **75**, pp. 3561-3568 (1994)
6. Koonin E.V. & Dolja V.V., *Crit. Rev. Biochem. and Mol. Biol.* **28**, pp. 375-430 (1993)
7. Schmitt C. et al., *Proc. Natl. Acad. Sci. USA.* **89**, pp. 5715-5719 (1992)
8. Morozov S.Y. et al., *J. Gen. Virol.* **72**, pp. 2039-2042 (1991)
9. Zhou H. & Jackson A.O., *Virology* **216**, pp. 367-379 (1996)
10. Quillet L. et al., *Virology* **172**, pp. 293-301 (1989)
11. Bleykasten et al., *J. Gen. Virol.* **77**, pp. 889-897 (1996)
12. Jupin I. et al., *Virology* **178**, pp. 273-280 (1990)
13. Sanger F. et al., *Proc. Natl. Acad. Sci. USA* **74**, pp. 5463-5467 (1977)
14. Fritsch C. et al., *J. Gen. Virol.* **46**, pp. 381-389 (1980)
15. Laemmli U.K., *Nature* **227**, pp. 680-685 (1972)
16. Schägger H. & von Jagow G., *Anal. Biochem.* **166**, pp. 368-379 (1987)
17. Lemaire O. et al., *Virology* **162**, pp. 232-235 (1988)
18. Niesbach-Klösgen U. et al., *Virology* **178**, pp. 52-61 (1990)

19. Hehn A. et al., *Virology* **210**, pp. 73-81 (1995)

20. Tamada T. et al., *J. Gen. Virol.* **70**, pp. 3399-3409 (1989)

21. Kozak M., *J. Cell. Biol.* **108**, pp. 299-241 (1989)

22. Pelletier J. & Sonenberg N, *Nature* **334**, pp. 320-325 (1988)

23. Tamada T. & Baba T., *Annals of the Phytopathological Society of Japan* **39**, pp. 325-332 (1973)

24. Kuszala M.& Putz C., *Annals of Phytopathology* **9**, pp. 435-446 (1977)

25. Keskin B., *Archiv für Mikrobiology* **49**, pp. 348-374 (1964)

26. Asher M.J.C., *Rhizomania* In *The sugar beet crop*, ed. D.A. Cooke and R.K. Scott, Chapman & Hall, London, pp. 312-338 (1993)

27. Richard-Molard M., *Rhizomanie* In *Institut français de la betterave industrielle. Compte-rendu des travaux effectués en* 1994, ITB, Paris pp. 225-229 (1995)

28. Henry C.M. et al, *Plant Pathology* **41**, pp. 483-489 (1992)

29. Grassi G. et al., *Phytopath. Medit.* **28**, pp. 131-139 (1989)

30. Merdinoglu D. et al., *Acad. Agric. Fr.* **79**, n° 6, pp. 85-98 (1993)

31. Scolten O.E. et al., *Archives of Virology* **136**, pp. 349-361 (1994)

32. Büttenr G. & Bürcky K., *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, Braunschweig Germany, August 21-24 (1990)

33. Whitney E.D., *Plant Disease* 73, pp. 287-289 1989)

34. Powell A.P. et al., *Science* **232**, pp. 738-743 (1986)

35. Fritchen J.H. & Beachy R.N., *Ann. Rev. Microbiol.* **47**, pp. 739-763 (1993)

36. Wilson T.M.A., *Proc. Natl. Acad. Sci. USA* **90**, pp. 3134-3141 (1993)

37. Gonsalves D. & Slightom J.L., *Seminars in Virology* **4**, pp. 397-405 (1993)

38. D'Halluin K. et al., *Biotechnology* **10**, pp. 309-314 (1992)

39. Kallerhof J. et al., *Plant Cell Reports 9*, pp. 224-228 (1990)

40. Ehlers U. et al., *Theoretical and Applied Genetic* **81**, pp. 777-782 (1991)

41. Kraus J. et al., *Field performance of transgenic sugar beet plants expresing BNYVV coat protein plants, Fourth International Congress of Plant Molecular Biology, Int. Soc. for Plant Molecular Biology*, Amsterdam (1994)

42. Maiss E. et al., *Proceedings of the Third Internaticnal Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*, Monterey, pp. 129-139 (1994)

43. Norris et al., *Plant Molecular Biology* **21**, pp. 895-906 (1993)

CLAIMS

1. Method for inducing resistance to a virus comprising a TGB3 sequence with the proviso that it is not the potato virus X, into a plant cell or a plant, comprising the following steps :

- preparing a nucleic acid construct comprising a nucleic acid sequence corresponding to at least 70% of the nucleic acid sequence of TGB3 of said virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant,
- transforming a plant cell with the nucleic acid construct, and possibly
- regenerating a transgenic plant from the transformed plant cell.

2. Method according to the claim 1, characterized in that the nucleic acid sequence of the nucleic acid construct corresponds to at least 90% of the nucleic acid sequence of TGB3 of said virus or its complementary cDNA.

3. Method according to the claim 1 or 2, characterized in that the virus is selected from the group consisting of the apple stem pitting virus, the blueberry scorch virus, the potato virus M, the white clover mosaic virus, the *Cymbidium* mosaic virus, the barley stripe mosaic virus, the potato mop top virus, the peanut clump virus and the beet soil-borne virus.

4. Method according to any of the preceding claims, characterized in that the plant cell is a stomatal cell.

5. Method according to any of the preceding claims, characterized in that the plant is selected from

the group consisting of apple, blueberry, potato, clover, orchid, barley or peanut.

6. Method according to claim 1 or 2, characterized in that the virus is BNYVV, the nucleic acid sequence of TGB3 of said virus is comprised between the nucleotide 3627 and 4025 of the 5' strand of genomic or subgenomic RNA 2 of the BNYVV and the plant is a beet, preferably a sugar beet (*Beta vulgaris*).

7. Method according to any of the preceding claims, characterized in that the regulatory sequence comprises a promoter sequence or a terminator sequence active in a plant.

8. Method according to claim 7 characterized in that the promoter sequence is a constitutive or a foreigner vegetal promoter sequence.

9. Method according to the preceding claim 7, characterized in that the promoter sequence is selected from the group consisting of 35S Cauliflower Mosaic Virus promoter, and/or the polyubiquitin Arabidopsis thaliana promoter.

10. Method according to any of the claim 7 to 9, characterized in that the promoter sequence is a promoter which is mainly active in the root tissue of plants such as the par promoter of the haemoglobin gene from Perosponia andersonii.

11. Transgenic plant resistant to a virus with the proviso that it is not the potato virus X, comprising a nucleic acid construct having a nucleic acid sequence corresponding to at least 70% of the nucleic acid sequence of TGB3 of said virus or its corresponding cDNA, being operably linked to one or more regulatory sequence(s) active in a plant.

12. Transgenic plant according to the claim 11, characterized in that the nucleic acid construct has a nucleic acid sequence corresponding to at least 90% of the nucleic acid sequence of TGB3 of said virus or its complementary cDNA.

13. Transgenic plant according to the claim 11 or 12, characterized in that the virus is selected from the group consisting of the apple stem pitting virus, the blueberry scorch virus, the potato virus M, the white clover mosaic virus, the *Cymbidium* mosaic virus, the potato virus X, the barley stripe mosaic virus, the potato mop top virus, the peanut clump virus and the beet soil-borne virus.

14. Transgenic plant according to the claims 11 to 13 being a plant selected from the group consisting of apple, blueberry, potato, clover, orchid, barley or peanut.

15. Transgenic plant according to the claims 11 or 12, characterized in that the transgenic plant being a beet, preferably a sugar beet (*Beta vulgaris*) the virus is BNYVV and the nucleic acid sequence of TGB3 of said virus is comprised between the nucleotide 3627 and 4025 of the 5' strand of genomic or subgenomic RNA 2 of BNYVV or its corresponding cDNA.

16. Transgenic plant according to any of the preceding claims 11 to 15, characterized in that the regulatory sequence comprises a promoter sequence and a terminator sequence active in a plant.

17. Transgenic plant according to any of the preceding claims 11 to 16, characterized in that the regulatory sequence(s) comprise a promoter sequence which is a constitutive or a foreigner vegetal promoter sequence.

18. Transgenic plant according to the claim 17, characterized in that promoter sequence is selected from the group consisting of 35S Cauliflower Mosaic Virus promoter, and/or the polyubiquitin Arabidopsis thaliana promoter.

19. Transgenic plant according to claim 17 or 18 characterized in that the promoter sequence is a promoter which is mainly active in root tissues such as the par promoter of the haemoglobin gene from Perosponia andersonii.

20. Transgenic plant tissue selected from the group consisting of fruit, stem, root, tuber, seed of a plant according to any of the preceding claims 11 to 19.

21. Reproducible structure obtained from a transgenic plant according to any of the preceding claims 11 to 19.

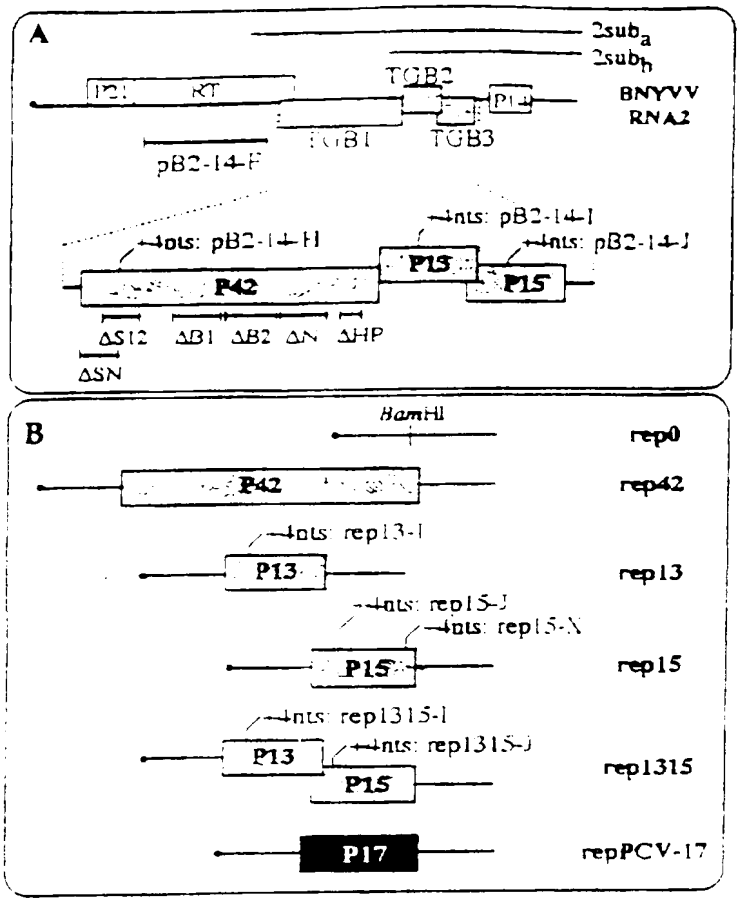


FIG.1

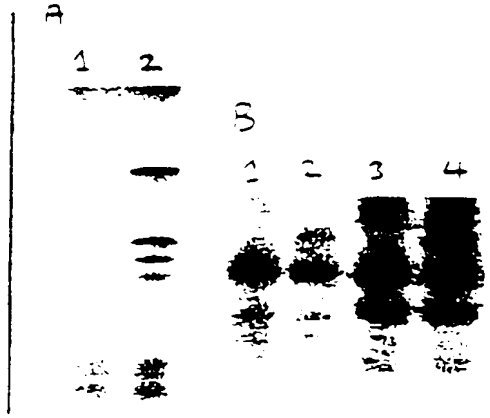


FIG.2

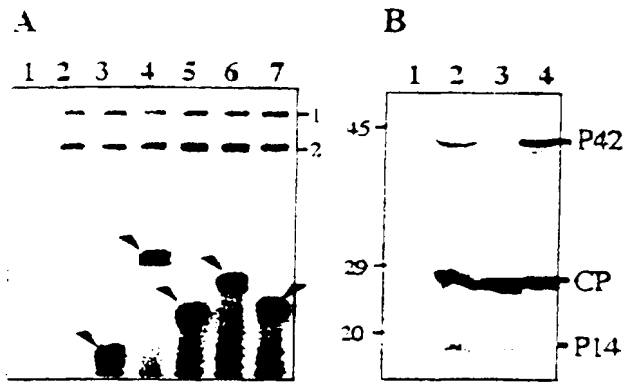


FIG.3

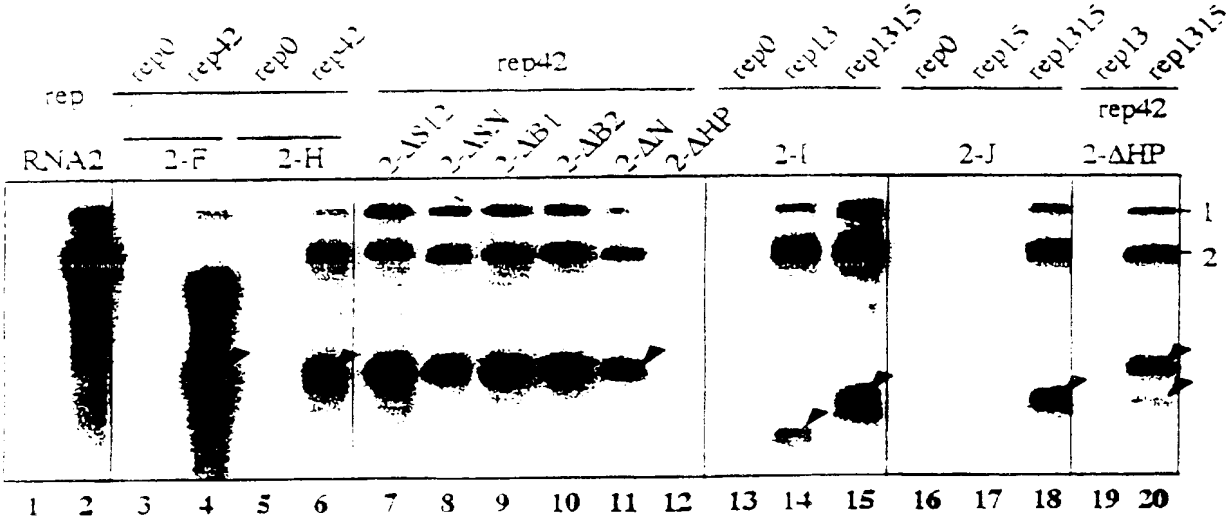


FIG.4

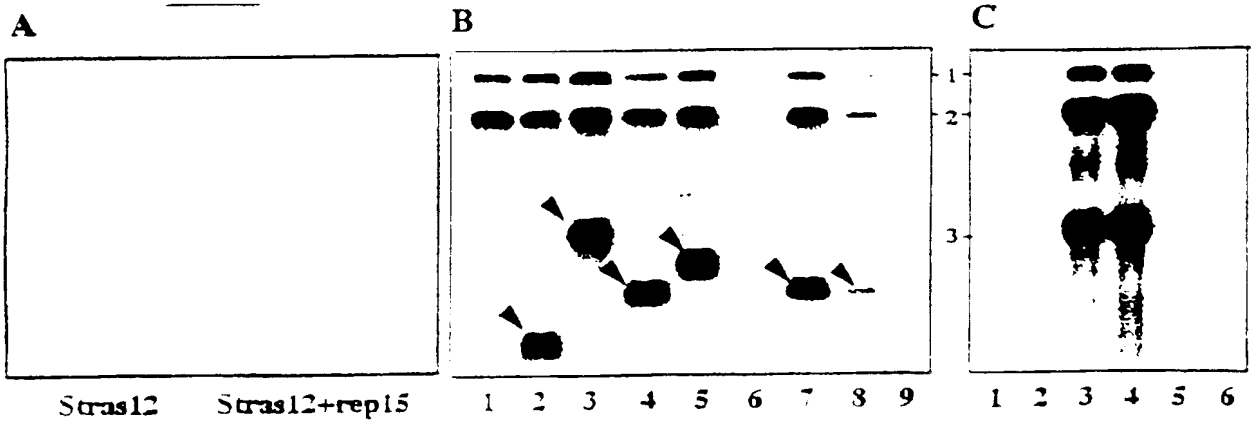


FIG.5

```
1/1                                                 31/11
ATG GTG CTT GTG GTT AAA GTA GAT TTA TCT AAT ATT GTA TTG TAC ATA GTT GCC GGT TGT
M   V   L   V   V   K   V   D   L   S   N   I   V   L   Y   I   V   A   G   C
61/21                                               91/31
GTT GTT GTC AGT ATG TTG TAC TCA CCG TTT TTC AGC AAC GAT GTT AAA GCG TCC AGC TAT
V   V   V   S   M   L   Y   S   P   F   F   S   N   D   V   K   A   S   S   Y
121/41                                              151/51
GCG GGA GCA ATT TTT AAG GGG AGC GGC TGT ATC ATG GAC AGG AAT TCG TTT GCT CAA TTT
A   G   A   I   F   K   G   S   G   C   I   M   D   R   N   S   F   A   Q   F
181/61                                              211/71
GGG AGT TGC GAT ATT CCA AAG CAT GTA GCC GAG TCC ATC ACT AAG GTT GCC ACC AAA GAG
G   S   C   D   I   P   K   H   V   A   E   S   I   T   K   V   A   T   K   E
241/81                                              271/91
GAC GAT GTT GAC ATA ATG GTA AAA AGG GGT GAA GTG ACC GTT CGT GTT GTG ACT CTC ACC
E   D   V   D   I   M   V   K   R   G   E   V   T   V   R   V   V   T   L   T
301/101                                             331/111
GAA ACT ATT TTT ATA ATA TTA TCT AGA TTG TTT GGT TTG GCG GTG TTT TTG TTC ATG ATA
E   T   I   F   I   I   L   S   R   L   F   G   L   A   V   F   L   F   M   I
361/121                                             391/131
TGT TTA ATG TCT ATA GTT TGG TTT TGG TAT CAT AGA TAA
C   L   M   S   I   V   W   F   W   Y   H   R   *
```

FIG.6

SEQ ID NO.1

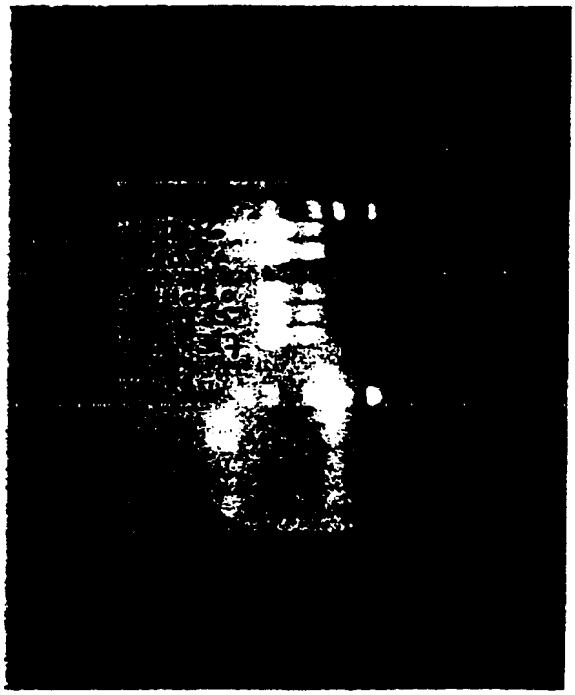


FIG. 7

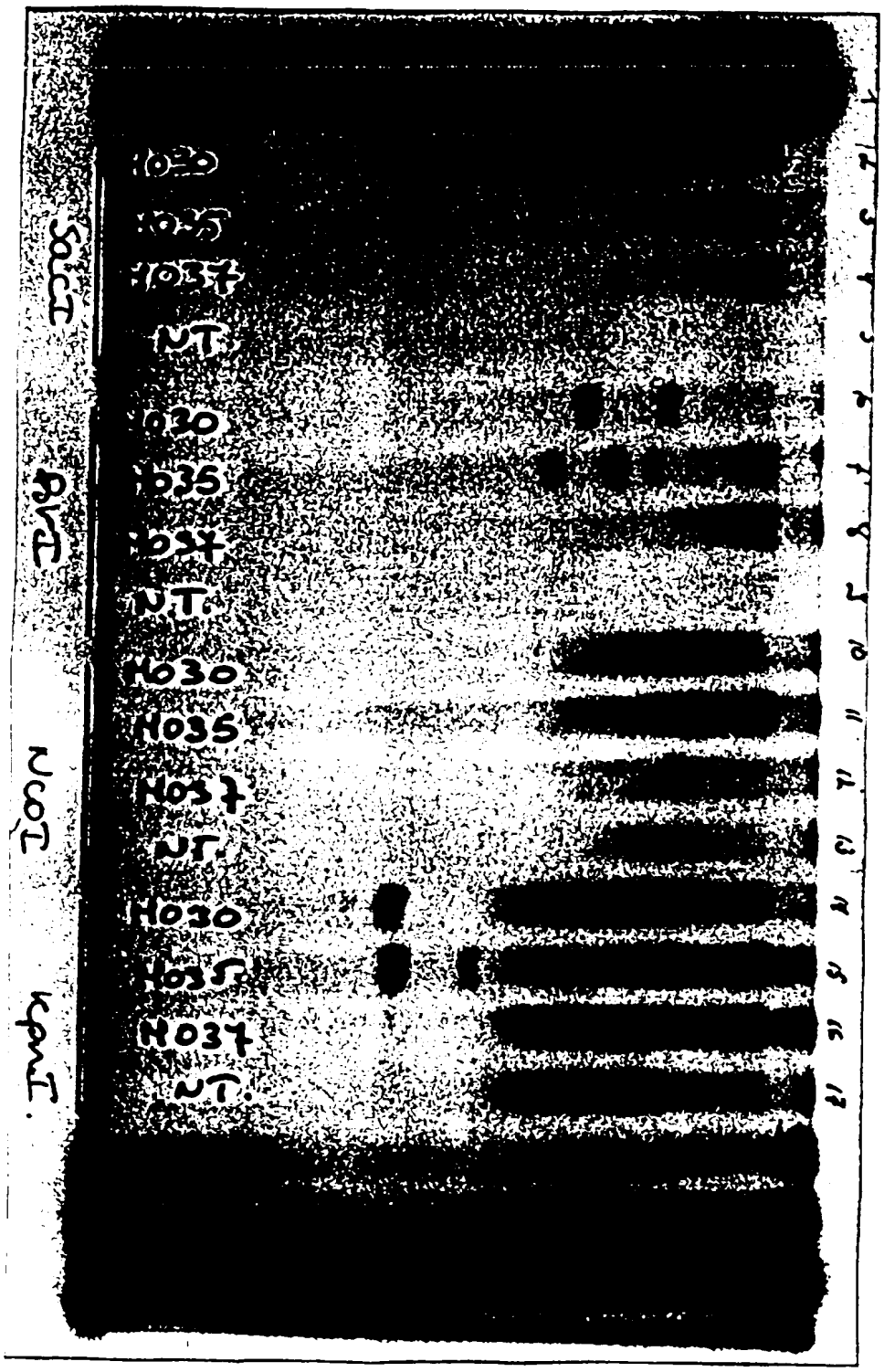


FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 97/00092

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H5/00 //C07K14/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	H. XU ET AL.: "Genetically engineered resistance to potato virus X in four commercial potato cultivars" PLANT CELL REP., vol. 15, 1995, pages 91-96, XP000617900 see abstract and page 95. ---	1,11
A	WO 91 13159 A (BIOSEM) 5 September 1991 cited in the application see Examples 15-18 and claims. --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1997	16. 12. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Yeats, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D.L. BECK ET AL.: "Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block" PROC. NATL. ACAD. SCI. USA, vol. 91, 1994, pages 10310-10314, XP002025561 see abstract. ---	1
A	D. GILMER ET AL.: "Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2" VIROLOGY, vol. 189, 1992, pages 40-47, XP002025562 cited in the application see abstract and Figure 1. -----	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 97/00092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9113159 A	05-09-91	FR 2658987 A	06-09-91
		FR 2658988 A	06-09-91
		AT 129745 T	15-11-95
		DE 69114275 D	07-12-95
		DE 69114275 T	13-06-96
		EP 0517833 A	16-12-92
		ES 2079647 T	16-01-96

PCT ORGANISATION MONDIALE DE LA PROPRIETE INTELLECTUELLE
Bureau international

DEMANDE INTERNATIONALE PUBLIEE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

(51) Classification internationale des brevets 5 : C12N 15/82, 5/10, A01H 5/00 C02N 15/40 | A2

(11) Numéro de publication internationale: WO 91/13159

(43) Date de publication internationale: 5 septembre 1991 (05.09.91)

(21) Numéro de la demande internationale: PCT/FR91/00170

(22) Date de dépôt international: 1er mars 1991 (01.03.91)

(30) Données relatives à la priorité:
90/02686 2 mars 1990 (02.03.90) FR
90/02687 2 mars 1990 (02.03.90) FR

(71) Déposant *(pour tous les Etats désignés sauf US)*: BIOSEM [FR/FR]; B.P. 1, Chappes, F-63720 Ennezat (FR).

(72) Inventeurs; et

(75) Inventeurs/Déposants *(US seulement)* : GERENTES, Denise [FR/FR]; 1, rue de la République, F-63170 Aubière (FR). PEREZ, Pascual [FR/FR]; 36, rue des Noyers, F-63110 BEAUMONT (FR). KALLERHOFF, Jean [FR/FR]; 56 bis, boulevard Gambetta, F-63400 Chamalières (FR). BEN TAHAR, Sophie [FR/FR]; 26, rue Vernemouze, F-63000 Clermont-Ferrand (FR). PERRET, Joël [FR/FR]; 26, chemin de Giroux, OPME, F-63540 Romagnat (FR).

(74) Mandataires: GUTMANN, Ernest etc. ; Ernest Gutmann Yves Plasseraud S.A., 67, boulevard Haussmann, F-75008 Paris (FR).

(81) Etats désignés: AT (brevet européen), BE (brevet européen), BG, CH (brevet européen), DE (brevet européen), DK (brevet européen), ES (brevet européen), FR (brevet européen), GB (brevet européen), GR (brevet européen), HU, IT (brevet européen), JP, LU (brevet européen), NL (brevet européen), RO, SE (brevet européen), SU, US.

Publiée
Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.

(54) Title: REGENERATION AND GENETIC TRANSFORMATION OF SUGAR BEET

(54) Titre: TRANSFORMATION GENETIQUE ET REGENERATION DE LA BETTERAVE SUCRIERE

(57) Abstract

A method for transforming plant cells of the species *Beta vulgaris*, wherein a dispersion or a suspension of crumbly white calluses is contacted with *Agrobacterium* containing a vector carrying a gene intended for insertion into the plant cells, followed by the coculture of the plant cells and the bacteria to produce transformed crumbly calluses, which are then regenerated into transgenic plants. Transgenic plants are also described which belong to the species *Beta vulgaris* and are resistant to infection by the beet necrotic yellow vein virus (BNYVV), said plants being stably transformed by a nucleic acid fragment which codes for at least a part of the capsid protein of BNYVV or for a derivative thereof.

(57) Abrégé

L'invention concerne un procédé de transformation de cellules végétales appartenant à l'espèce *Beta vulgaris* caractérisé en ce qu'il comprend la mise en contact d'une dispersion de cals blancs friables, ou d'une suspension de cals blancs friables avec *Agrobacterium* contenant un vecteur portant un gène destiné à être introduit dans les cellules végétales, suivie de coculture des cellules végétales et des bactéries pour donner lieu à des cals friables transformés. Les cals transformés sont ensuite régénérés en plantes transgéniques. L'invention concerne également des plantes transgéniques appartenant à l'espèce *Beta vulgaris* et résistantes à l'infection par le virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV), lesdites plantes étant transformées d'une manière stable par un fragment d'acide nucléique, ledit fragment codant pour au moins une partie de la protéine de capside du BNYVV ou pour un dérivé de cette protéine.

UNIQUEMENT A TITRE D'INFORMATION

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

AT	Autriche	**ES**	Espagne	**MG**	Madagascar
AU	Australie	**FI**	Finlande	**ML**	Mali
BB	Barbade	**FR**	France	**MN**	Mongolie
BE	Belgique	**GA**	Gabon	**MR**	Mauritanie
BF	Burkina Faso	**GB**	Royaume-Uni	**MW**	Malawi
BG	Bulgarie	**GN**	Guinée	**NL**	Pays-Bas
BJ	Bénin	**GR**	Grèce	**NO**	Norvège
BR	Brésil	**HU**	Hongrie	**PL**	Pologne
CA	Canada	**IT**	Italie	**RO**	Roumanie
CF	République Centrafricaine	**JP**	Japon	**SD**	Soudan
CG	Congo	**KP**	République populaire démocratique de Corée	**SE**	Suède
CH	Suisse			**SN**	Sénégal
CI	Côte d'Ivoire	**KR**	République de Corée	**SU**	Union soviétique
CM	Cameroun	**LI**	Liechtenstein	**TD**	Tchad
CS	Tchécoslovaquie	**LK**	Sri Lanka	**TG**	Togo
DE	Allemagne	**LU**	Luxembourg	**US**	Etats-Unis d'Amérique
DK	Danemark	**MC**	Monaco		

BNSDOCID: <WO 9113159A2>

TRANSFORMATION GENETIQUE ET REGENERATION DE LA BETTERAVE SUCRIERE

L'invention concerne un procédé de transformation génétique de cellules végétales appartenant à l'espèce Beta vulgaris, suivie éventuellement par une étape de régénération de cellules transformées en plante entière.

L'invention concerne également les cellules transformées, les bourgeons, les plantes et les graines transgéniques susceptibles d'être produits par ce procédé.

L'invention concerne en outre, des plantes transgéniques appartenant à l'espèce Beta vulgaris, résistantes à l'infection par le virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV), exprimant la protéine de capside du BNYVV, ou un dérivé de cette protéine.

Les références bibliographiques apparaissant dans la description de l'invention sont répertoriées sous forme de bibliographie.

L'obtention de plantes transgéniques met en oeuvre le transfert du fragment d'ADN sélectionné dans la cellule végétale, la sélection des cellules transformées de façon stable et la régénération de plantes entières à partir des cellules sélectionnées transformées.

Le problème technique qui s'est posé lors de l'élaboration de la présente invention était de trouver une méthode de transformation de cellules de betterave présentant une fréquence de transformation élevée qui pouvait être associée avec succès à une méthode de régénération de plante transgénique. A ce jour, une telle méthode de transformation et de régénération n'a pas été décrite, empêchant la production de betteraves transgéniques présentant des

caractéristiques agronomiquement intéressantes, telle que la résistance à la rhizomanie.

Actuellement, il existe deux grandes voies de transfert d'ADN dans les cellules végétales.

La première voie est une voie physico-chimique (électroporation, microinjection, polycations, canon à particules). Chez la betterave, des cellules transformées de manière stable ont été sélectionnées après électroporation d'un gène codant pour la résistance à la kanamycine dans les protoplastes (Lindsey et al, 1989).

Cependant, ce procédé n'a jamais pu conduire à la création de betteraves transgéniques puisque la régénération de plantes à partir de protoplastes n'a pu être obtenue chez cette espèce.

La deuxième voie de transfert d'ADN est une voie biologique utilisant comme vecteur une bactérie du sol : Agrobacterium tumefaciens ou rhizogenes.

Différentes souches de ces deux espèces ont été utilisées avec succès pour la transformation de cellules de betteraves. Ces cellules transformées ont donné naissance soit à des tumeurs avec Agrobacterium tumefaciens (Krens et al, 1988) soit à des racines avec Agrobacterium rhizogenes (Yacoub et al, 1987). Ces tissus transformés (racines et tumeurs) n'ont jamais permis à ce jour la régénération de plantes chez la betterave. Ceci vient du fait que ces deux phénotypes sont les résultats de l'intégration dans le génome de la cellule non seulement du fragment d'ADN désiré mais aussi d'un fragment d'ADN de la bactérie qui perturbe l'équilibre hormonal de la cellule (Akiyoshi et al, 1983). Pour pallier ces problèmes, ces gènes ont été délétés, donnant de nouvelles souches d'Agrobacterium dites souches désarmées.

L'utilisation des souches d'Agrobacterium désarmées implique l'association d'un système sélectif pour la transformation de cellules végétales. Le succès dans l'obtention de transformants est étroitement lié à la mise au point d'un bon système sélectif. Le gène sélectif le plus utilisé dans ce domaine est celui provenant du transposon Tn5 d'Escherichia coli (Rothstein et al, 1981) codant pour la néomycine phosphotransférase (NPT II) qui confère la résistance à la kanamycine (An et al, 1985).

La plupart des vecteurs plasmidiques utilisés dans ces souches désarmées contiennent dans l'ADN transférable, outre le gène désiré, le gène NPT II, sous le contrôle de signaux de transcription végétaux (Bevan, 1984 ; An, 1986). Le système de sélection comprend d'une part le gène conférant la résistance, et d'autre part, l'agent sélectif. Ceci implique de déterminer les concentrations d'agent sélectif permettant à la fois de tuer les cellules non transformées et de laisser croître les cellules transformées. Les seuls travaux publiés concernant la sélection de cellules de betterave après transformation d'explants pluricellulaires par Agrobacterium font appel à un autre système sélectif : hygromycine B / hygromycine B phosphotransferase (Harpster et al, 1988).

La transformation par Agrobacterium nécessite dans un premier temps le choix d'un type de cellule ou d'un type d'explant qui va faire l'objet de la transformation. Par exemple, des explants tels que des hypocotyles, des morceaux de feuilles (Krens et al, 1988) peuvent être transformés par Agrobacterium.

La fréquence de transformation peut varier selon le type de cellule ayant fait l'objet de la

transformation, cette variabilité étant souvent d'une nature imprévisible.

Lors de la production d'une plante transgénique, la transformation est suivie par un procédé de régénération.

L'efficacité d'obtention des plantes transgéniques dépend de la fréquence de régénération de plantes à partir de cellules transformées d'une part, et la fréquence de transformation des cellules d'autre part.

Par exemple, la transformation de tronçons de pétioles de betterave a donné des cals transformés sélectionnés sur kanamycine. Cependant, il n'a jamais été possible de régénérer des plantes à partir de ces cals transformés issus de pétioles, bien que la régénération d'embryons somatiques et de bourgeons à partir de ce type de cal dans l'état non-transformé ait été reportée (Tetu et al, 1987). Cet échec n'est pas surprenant si on prend en compte la faible fréquence de régénération décrite.

Ceci traduit bien les problèmes de régénération, à partir d'explants, de plantes non-transformées, rencontrés depuis longtemps chez la betterave sucrière (Ritchie et al, 1989). Plusieurs auteurs ont décrit la régénération directe de bourgeons adventifs à partir de fragments de pétioles de plantes en multiplication végétative (Detrez et al, 1988 ; Freytag et al, 1988). Bien que ce phénomène se soit avéré reproductible dans des conditions appliquées par les inventeurs, la fréquence s'est révélée beaucoup trop faible pour être associée à la transformation. D'autres part, il semble que ces néoformations proviennent de massifs cellulaires non accessibles à la bactérie et apparemment peu sensibles à un agent sélectif.

Une autre technique de régénération de plantes non transformées à partir de cellules de betteraves à sucre est celle décrite par Saunders et al (1986). Elle met en jeu dans un premier temps, l'induction de cals friables indépendants d'hormones et initiés probablement à partir des cellules épidermiques du limbe, puis dans un deuxième temps, la régénération de bourgeons et d'embryons somatiques à partir de ces cals. Cette technique a été facilement reproductible sur plusieurs variétés de betteraves sucrières. Un des intérêts de ce processus est la possibilité d'obtenir aisément des suspensions cellulaires à partir de cals friables dont le potentiel organogène peut être entretenu pendant quelques mois.

Les inventeurs ont découvert que ce matériel organogène, c'est-à-dire les cals friables, peut être utilisé, dans des conditions précises, pour la transformation par Agrobacterium tumefaciens.

Il y a encore peu de temps la transformation de suspensions cellulaires ne paraissait pas concevable selon le dogme établi que le transfert d'ADN par Agrobacterium dans une cellule végétale nécessitait des lésions cellulaires. Cependant, des auteurs ont reporté la transformation de cellules en suspension chez le tabac et la carotte (AN, 1985 ; Scott et Draper, 1987). A ce jour, la transformation de cellules en suspension chez la betterave n'a pas été décrite. Par ailleurs, il est à noter que les méthodes de transformation qui s'appliquent avec succès à une espèce végétale ne peuvent pas être étendues systématiquement à d'autres espèces. Les conditions de transformation, les matériels de départ et les milieux de culture sont des paramètres variables spécifiques à chaque espèce.

En ce qui concerne la transformation et la régénération de betteraves transgéniques résistantes à la rhizomanie, tout progrès a été empêché, par le manque d'un procédé de production fiable.

Le virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV) est un virus à composants multiples, constitué de particules virales à symétrie hélicoïdale, contenant quatre types de RNA simple brin, de polarité positive (Putz, 1987). Ce virus est disséminé par un champignon du sol, Polymixa betae, qui parasite les cellules superficielles des radicelles de chénopodiacées, dont fait partie la betterave à sucre. Cette chénopodiacée bisannuelle reste à l'état de rosette la première année en donnant une racine charnue et sucrée, et monte à graine la deuxième année après vernalisation. La persistance de la maladie dans le sol est due aux kystes formés par le champignon (Tamada, Baba, 1973).

Il est connu que le virus se développe essentiellement dans la partie racinaire (pivot et racines secondaires). Le symptôme principal de la maladie consiste en une prolifération du chevelu racinaire (d'où le nom de rhizomanie). Mais le nom du virus provient en fait de symptômes plus tardifs visibles dans la partie végétative à savoir : nécroses et jaunissement des nervures dus au fait que le virus se développe surtout au niveau des vaisseaux racinaires, en perturbant ainsi le métabolisme de toute la plante (Sallé et al, 1986).

Plusieurs auteurs ont reporté que le virus n'est détecté dans la partie aérienne que très rarement alors qu'il est présent en grande quantité au niveau des racines et du pivot (Putz, 1977 ; Ziegler et al, 1985).

Le contrôle des maladies virales des végétaux reste problématique, malgré l'avènement du génie génétique végétal.

Abel et al (1986) ont introduit un gène codant pour la protéine de capside du virus de la mosaïque du tabac (VMT) dans le génome des plantes naturellement sensibles à ce virus. Cette manipulation génétique a provoqué un retard du développement de la maladie chez les plantes transgéniques. D'autres réalisations du même type ont été reportées avec d'autres virus ;

- Alfalfa Mosaic Virus, et Tobacco Rattle Virus, sur tabac (Van Dun et al, 1987),
- Alfalfa Mosaic Virus sur tabac (Loesch Fries et al, 1987),
- Alfalfa Mosaic Virus sur tabac et tomate (Tumer et al, 1987).

Deux équipes ont créé des plantes transgéniques exprimant des gènes codant pour des ARN antisens, complémentaires de l'ARN codant pour la protéine de capside, et ils montrent que la résistance est beaucoup moins importante que celle conférée par la protéine de capside (Cuozzo et al, 1988 ; Hemenway et al, 1988).

Enfin, d'autres laboratoires ont créé des plantes transgéniques exprimant des gènes codant pour des ARN satellites. Cette stratégie a été adoptée par Gerlach et al (1987) pour le virus des tâches annulaires du tabac, et par Harrison et al (1987) pour le virus de la mosaïque du concombre (CMV).

Aucun satellite n'étant connu pour le BNYVV, les inventeurs ont envisagé de faire produire aux cellules végétales transformées soit des ARN antisens, soit des ARN sens codant pour des protéines virales normales, mutées ou délétées, susceptibles d'inhiber le développement du virus.

Le procédé de transformation de l'invention implique :

- la transformation de suspensions cellulaires habituées, donnant des cals et suspensions cellulaires transformés ;

- la transformation de suspensions cellulaires régénérantes donnant des cals, des suspensions cellulaires et des plantes transformés ;

- la transformation de cals friables en dispersion donnant des cals, des suspensions cellulaires et des plantes transformés.

Plus particulièrement, la présente invention concerne un procédé de transformation de cellules végétales appartenant à l'espèce Beta vulgaris caractérisé en ce qu'il comprend la mise en contact d'une dispersion de cals blancs friables dans un milieu de culture cellulaire végétale liquide contenant 0 à environ 3.0 mgL^{-1} d'une cytokine, ou d'une suspension de cals blancs friables dans un milieu de culture cellulaire végétale liquide contenant environ 0.1 à environ 3.0 mgL^{-1} d'une cytokinine, avec Agrobacterium contenant un vecteur portant un gène destiné à être introduit dans les cellules végétales, suivie de coculture des cellules végétales et des bactéries pour donner lieu à des cals friables transformés. Selon un mode de réalisation préféré de l'invention, ce procédé de transformation comprend les étapes successives suivantes :

I) induction de cals blancs friables à partir d'explant ;

II) dispersion des cals dans un milieu de culture cellulaire végétale liquide contenant 0 à environ 3.0 mgl^{-1} d'une cytokinine, ou obtention d'une suspension cellulaire à partir des cals dans un milieu

de culture cellulaire végétale liquide contenant environ 0.1 à environ 3.0 mgl^{-1} d'une cytokinine ;

III) mise en contact de la dispersion, ou de la suspension, avec Agrobacterium tumefaciens contenant un vecteur portant un gène destiné à être introduit dans les cellules végétales, suivie de coculture des cellules végétales et des bactéries ;

IV) lavage des cellules végétales pour éliminer les bactéries et sélection des cellules transformées sur un milieu sélectif ;

V) culture des cellules transformées sélectionnées pour obtenir des cals friables transformés.

La première étape de la transformation est l'induction de cals blancs friables à partir d'explants de betterave. Les explants qui peuvent servir dans cette étape peuvent être par exemple des disques de feuilles, des tronçons de pétioles, etc. De préférence, les explants sont des morceaux de jeunes feuilles prélevées d'une plante âgée de moins de trois mois.

Par exemple, après germination de graines de betterave d'environ un mois, de jeunes feuilles de 3 à 5 cm de long sont prelevées de chaque plante et sont soumises à une étape de désinfection et rinçage. Chaque feuille est ensuite découpée en petits morceaux de 0,25 cm^2 à 1.0 cm^2. Les feuilles peuvent être prélevées de la plante jusqu'à deux mois environ après les premiers prélèvements. Après cette période, l'aptitude de régénération des feuilles diminue. Les disques de feuilles sont ensuite mis en culture sur un milieu de culture cellulaire végétale contenant de 0,1 à 5,0 mgl^{-1} cytokinine. De préférence, la cytokinine est présente à raison d'environ 1.0 mgl^{-1} et peut être, par exemple, la 6-benzylaminopurine (BAP), la zéatine,

ou la kinétine. La BAP est particulièrement préférée. Le milieu de culture cellulaire végétale est avantageusement le milieu de Murashige et Skoog (1962), dit milieu M.S. Les explants sont cultivés à 30°C environ pendant 30 jours dans l'obscurité, et ensuite ils sont sortis en chambre de culture avec une photopériode de 18/24 H par exemple à environ 25°C le jour et 20°C la nuit.

De 4 à 10 semaines après la mise en culture, des cals blancs friables apparaissent autour, sur ou sous les explants foliaires.

L'étape suivante du procédé de transformation est la production d'une suspension cellulaire à partir des cals, ou d'une dispersion des cals dans un milieu de culture liquide. Cette suspension ou dispersion sera utilisée ultérieurement pour la transformation.

La suspension cellulaire est obtenue par mise en culture des cals, 4 à 6 semaines après leur apparition dans un milieu de culture liquide additionné de 0,1 à 3,0 mgl^{-1} d'une cytokinine, par exemple de la BAP. Cette étape de culture dure 2 à 3 semaines et est effectuée sous agitation. Le milieu de culture est avantageusement le milieu M.S. Un milieu particulièrement préféré est le milieu M.S additionné de 1 mgl^{-1} BAP. Ce milieu sera appelé le milieu MSB1 dans ce qui suit. Sa composition est indiquée dans le tableau 3 (voir exemple 9).

La suspension cellulaire s'établit en 2 ou 3 semaines. Chaque suspension est ensuite repiquée toutes les 3 semaines par filtration des suspensions sur trois tamis empilés, donnant lieu à trois fractions (par exemple > 1 mm ; > 500 μm, > 100 μm). Une partie de chaque fraction est remise en suspension dans du milieu liquide, par exemple le milieu MSB1, et ces nouvelles suspensions sont agitées. L'observation

des différentes suspensions permet de distinguer la présence de deux types cellulaires, notamment :

- type habitué (A) : suspension fine, verte à croissance rapide, et qui régénère ponctuellement des formations vitrifiées se développant difficilement ;

- type noduleux (C) : suspension d'agrégats compacts jaunâtres, à croissance plus lente, régénère plus fréquemment que la première, des structures embryonnaires compactes se développant assez bien. Ce type est aussi connu sous le nom "type régénérant".

En revanche, la production d'une dispersion des cals s'effectue par dispersion des cals apparus depuis 2 à 6 semaines sur les explants, dans un milieu de culture liquide contenant 0 à 3,0 mgl^{-1} cytokinine, par exemple le milieu MSB1. Les deux types cellulaires, habitué et noduleux, peuvent aussi être observés dans les dispersions. Les cals en dispersion peuvent être examinés avant la transformation dans le but de séparer les deux types cellulaires, c'est-à-dire noduleux et habitué. Un examen visuel des cals permet de repérer les deux types qui sont ensuite enlevés du milieu avec une pince et redispersés. Les suspensions cellulaires soigneusement initiées à partir de dispersions de cals blancs friables noduleux se sont avérées être le matériel idéal pour optimiser l'efficacité de la transformation.

Chacun des deux types cellulaires peuvent être soumis à la transformation mais il est préférable de transformer le type noduleux si la régénération de la plante est désirée ultérieurement.

La suspension cellulaire ou la dispersion des cals est ensuite mise en contact avec la bactérie Agrobacterium tumefaciens.

Le protocole de transformation est le même, qu'il soit effectué sur des suspensions cellulaires ou sur

les dispersions. Un échantillon des bactéries dans du milieu frais est ajouté à la suspension ou à la dispersion des cellules de betterave. La coculture des cellules végétales et des bactéries se fait à l'obscurité pendant trois jours environ, en chambre de culture.

L'Agrobacterium utilisé dans la transformation contient un vecteur portant un gène destiné à être introduit dans les cellules de betterave. Les souches utilisées par les inventeurs contiennent des vecteurs binaires portant le gène d'intérêt. Les trois souches d'Agrobacterium tumefaciens désarmées utilisées dans le travail décrit ici sont LBA 4404 (Hoekema et al, 1983 ; EHA 101 (Hood et al, 1986) ; C58'3 (Dale et al, 1989). Bien évidemment, le gène d'intérêt est placé sous le contrôle de signaux régulateurs appropriés, par exemple un promoteur permettant son expression dans la cellule végétale et, le cas échéant, dans la plante transgénique régénérée. Le promoteur peut être choisi pour permettre l'expression spécifique du gène dans une certaine partie de la plante, ou à un certain stade de son développement. En revanche, un promoteur constitutif peut être utilisé, donnant lieu à l'expression ubiquitaire du gène introduit.

Comme gène, on peut citer des gènes codants pour des caractères agronomiques intéressants, par exemple la résistance aux herbicides, aux insectes et aux virus, ou encore un gène induisant la stérilité mâle. La résistance aux herbicides peut être conférée, par exemple par le type de gène décrit par De Block et al (1987) et par Bedbrook et al (1988). Un gène susceptible de conférer une résistance aux insectes est le gène de la protéine crystalline de B. thiuringiensis (Perlak et al (1990) ; Vaeck et al (1987) ; Fischoff D, et al (1987)) La stérilité mâle

peut être induite par un gène codant pour un ribonucléase, tel que celui décrit par Mariani et al (1990). La résistance aux virus peut parfois être induite par le gène codant pour la protéine de capside du virus en question. Par exemple, la résistance au virus BWYV peut être conférée par le gène décrit par Gielen et al (1990). Par ailleurs, une protection contre le BNYVV, responsable de la rhizomanie, peut également être induite par ce même type de gène. Selon le procédé de l'invention, ce gène codant pour cette protéine de capside peut être introduit dans des cellules de betteraves, conférant ainsi la résistance à la rhizomanie. Les vecteurs décrits dans ces exemples peuvent être utilisés pour l'introduction de ces gènes.

Le vecteur porte également un gène codant pour une protéine permettant la sélection des transformants, par exemple la néomycine phosphotransférase (NPT II) qui confère la résistance à la kanamycine. De plus, un gène "reporteur" peut être introduit dans le vecteur afin de pouvoir confirmer le caractère transformé du tissu végétal. Un exemple d'un tel gène reporteur est le gène uid A d'E. coli codant pour l'enzyme β-glucuronidase. Le dosage de l'activité enzymatique de cette protéine se fait facilement avec des substrats chromogènes ou fluorescents.

Après la coculture des cellules de betteraves et des bactéries, une étape de lavage avec un agent bactériostatique est effectué pour éliminer les bactéries. Comme agent bactériostatique, la cefotaxime peut être utilisée. Le lavage peut être effectué en deux étapes, par exemple une première fois avec le milieu MSB1 contenant 600 mgl^{-1} cefotaxime, puis une

deuxième fois dans du milieu MSB1 contenant 300 mgl^{-1} cefotaxime.

Ensuite, la sélection des cellules transformées est effectuée grâce au gène codant pour la résistance à l'agent sélectif. Les cellules lavées sont donc mises en culture pendant une quinzaine de jours sur un milieu contenant l'agent sélectif, par exemple la kanamycine et l'agent bactériostatique. Les cellules végétales sont ensuite repiquées sur du milieu frais.

Trois à huit semaines après la coculture, des cals blancs apparaissent. Ces cals transformés présentent les deux types cellulaires, noduleux et habitué.

Les cals transformés ainsi obtenus ont, comme caractéristique physique principale, qu'ils peuvent être dispersés en liquide par une agitation relativement douce et celà, dès qu'ils ont une taille de 3 à 5 mm. D'une manière surprenante, ces cals transformés conservent la nature blanche, friable et régénérante qu'ils possédaient avant la transformation, et s'avèrent donc être un matériel approprié pour la régénération de plantes transgéniques. Il est à noter que la transformation effectuée sur explants, et non sur cals (Harpster et al, 1988), donne lieu à des cals transformés très compacts qui ne présentent pas de caractère friable et qui ne sont pas régénérants.

Quand ils sont suffisamment développés, les cals transformés de l'invention sont repiqués sur un milieu MSB1 avec de la céfotaxime et éventuellement de la kanamycine.

D'une manière surprenante, il a été constaté qu'un mois après le clonage des cals transformés, la kanamycine et la céfotaxime peuvent être supprimés du milieu sans que la bactérie ne se développe. Ceci peut

présenter un avantage pour l'étape de régénération, les cellules n'étant plus en contact avec les antibiotiques.

Après l'obtention des cals friables transformés, la régénération de la plante transgénique peut être initiée, en commençant par la régénération de bourgeons et/ou d'embryons transgéniques.

Le procédé de régénération de bourgeons et/ou d'embryons transgéniques selon l'invention est caractérisé par l'obtention de cals friables transformés selon le procédé décrit ci-dessus, suivie du repiquage des cals friables transformés sur un milieu de culture, par exemple le milieu M.S, contenant 0 à 3,0 mgl^{-1} d'une cytokinine et éventuellement un agent bactériostatique et un agent sélectif. Comme cytokinine, on peut citer la zéatine, la kinétine et la BAP, par exemple à une concentration de 1 mgl^{-1}. La BAP est particulièrement préférée.

Des bourgeons et/ou embryons régénèrent sur certains des cals après des délais variant d'une semaine à plusieurs mois.

A partir de ces bourgeons et/ou embryons transgéniques, il est possible, selon l'invention, de régénérer des plantes par repiquage des bourgeons et/ou embryons sur un milieu de culture tel que le milieu M.S contenant un cytokinine à faible concentration, par exemple entre 0,05 et 0,15 mgl^{-1}, de préférence 0,1 mgl^{-1}. La BAP est préférée dans cette étape. Les structures régénérées commencent alors à développer des feuilles et elles sont remises en multiplication végétative en pots ou boîtes. Les bourgeons les plus développés sont alors mis sur un milieu d'enracinement tel que le milieu MS contenant de l'acide naphtalène-acétique, par exemple 1 mgl^{-1}.

Les racines apparaissent 2 à 6 semaines après. Les plantes peuvent alors être acclimatées en serre.

L'invention concerne également la production de graines à partir des plantes transgéniques par vernalisation de ces plantes transgéniques et récolte des graines après floraison.

L'invention concerne également les cals friables transformés, les bourgeons et/ou embryons transformés, les plantes transgéniques et les graines de ces plantes susceptibles d'être produits selon les procédés décrits ci-dessus.

La présente invention concerne également la transformation génétique et la régénération de la betterave à sucre (Beta vulgaris ssp saccharifera) dans le but de créer des plantes résistantes à la rhizomanie.

Plus particulièrement, les inventeurs ont transformé des cellules végétales par des séquences codant pour la protéine de capside du BNYVV, ou pour des variantes de celle-ci.

La mise en oeuvre de cet aspect de l'invention a consisté à :

- construire par les techniques de recombinaison génétique in vitro, des gènes artificiels, potentiels de résistance au virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV : Beet Necrotic Yellow Vein Virus) provoquant la rhizomanie,
- transférer de façon stable ces gènes de résistance dans des betteraves à sucre.

Le gène codant pour la protéine de capside du BNYVV a été localisé et séquencé (Bouzoubaa et al, 1986). Cependant, il ne pouvait pas être prévu que l'expression de cette protéine dans des cellules de betterave inhiberait le développement du virus. Le virus étant transmis par le champignon du sol Polymixa

betae, la voie d'infectabilité n'est pas comparable à celle des virus pour lesquels la protéine de capside s'est montré protectrice. D'autre part, la protéine de capside du BNYVV est codée par l'extrémité 5'du ARN2, ce qui veut dire que la protéine peut être traduite dans la cellule infectée immédiatement après l'infection. Pour d'autres virus, la protéine de capside est codée par un ARN subgénomique et est donc produite plus tard dans le cycle de l'infection. Pour ces raisons, l'effet protecteur présenté par la protéine de capside pour d'autres virus végétaux ne pouvait pas être prévu chez le BNYVV.

De plus, les inventeurs ont constaté plusieurs phénomènes inattendus : premièrement, il a été observé que des cellules transformées par une même séquence codant pour la protéine de capside de 22 Kd (nucléotides 145 à 708) et au moins une partie de la protéine de 75 Kd qui est composée de la protéine de 22 Kd fusionnée avec la protéine 54 Kd (nucléotides 709 à 2218), donne lieu à l'expression de deux protéines dans la même cellule, c'est-à-dire la protéine de 22 Kd et une protéine chimérique composée de la protéine de 22 Kd additionnée de la partie de la protéine de 75 Kd codée par la séquence transformante. Cette expression est le résultat du phénomène de "readthrough", le codon "stop" à la fin de la protéine de 22 Kd étant parfois supprimé dans la cellule par des tRNA suppresseurs. Il a été constaté que le taux d'expression de la protéine chimérique de "readthrough" est particulièrement élevé, et pourrait jouer un rôle dans la résistance conférée à la cellule exprimant les deux protéines à la fois.

Deuxièmement, les inventeurs ont observé que dans des plantes transgéniques les protéines exprimées selon l'invention sont exprimées spécifiquement dans

les racines, et ce malgré l'utilisation de promoteurs constitutifs.

Ce phénomène est totalement inattendu et présente plusieurs avantages pour la plante. Tout d'abord, les cellules cibles du BNYVV sont spécifiquement protégées. De plus, l'absence d'expression de la protéine de capside et de ses dérivés dans les parties aériennes de la plante, partie qui n'est pas susceptible d'être infectée par le BNYVV, représente une économie énergique importante pour la plante. Par ailleurs, l'expression dans les racines, à l'exclusion de toute expression dans d'autres parties de la plante, est un phénomène qui n'aurait pas pu être obtenu en utilisant un promoteur dit "spécifique" pour les racines. Ce genre de promoteur conduit, en fait, à une expression plus importante dans les racines, et une expression faible dans les autres parties de la plante. Des expériences, effectuées par les inventeurs, utilisant un gène marqueur (GUS) ont démontré que l'expression spécifique n'est pas dû à une mauvais fonctionnement du promoteur, puisque le produit d'expression du gène GUS est exprimée dans toutes les parties de la plante, lorsqu'elle est sous le contrôle de ces mêmes promoteurs. L'expression spécifique pourrait être le résultat de l'instabilité de la protéine dans les parties aériennes de la plante, ou à une mauvaise traduction. Ces expériences montrent que le promoteur ne semble pas jouer de rôle dans l'expression spécifique.

Il a également été constaté que le promoteur 35S a une efficacité de transcription de 30 à 50 fois plus élevée que le promoteur Nos dans des cellules de betterave.

L'invention concerne des plantes transgéniques produisant :

- la protéine de capside du BNYVV,

- des protéines de capside modifiées définies ci-dessous (acides aminés supplémentaires, acides aminés permutés, acides aminés délétés), qui conservent l'effet protecteur vis-à-vis du BNYVV

- des ARN sens et des ARN antisens de différentes tailles et dirigés contre différentes régions de l'ARN2 du BNYVV.

Plus particulièrement, cet aspect de l'invention concerne une plante transgénique appartenant à l'espèce Beta vulgaris et résistante à l'infection par le virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV), ladite plante étant transformée d'une manière stable par un fragment d'acide nucléique, dont le produit d'expression est capable de conférer ladite résistance, ledit fragment étant dérivé de l'extrémité 5' de l'ARN2 génomique ou subgénomique du BNYVV, ou du cADN correspondant, ce fragment codant pour au moins une partie des protéines codées par les nucléotides 145 à 3285 de la séquence sauvage de l'ARN2, et étant sous le contrôle d'un promoteur permettant l'expression du fragment dans les cellules de la plante et étant dans l'orientation sens ou antisens.

Dans le contexte de l'invention, un fragment "dérivé de l'extrémité 5' de l'ARN2 du BNYVV" signifie le cADN correspondant, ou une variante capable d'hybrider avec celui-ci dans des conditions non stringentes, ou dont le produit d'expression présente au moins 80 % d'homologie. Il est important que les variantes présentent la propriété de pouvoir inhiber l'infection par le BNYVV dans des cellules l'exprimant. Par "inhiber", il faut comprendre une réduction et un retard significatifs de l'apparition des symptômes de l'infection et de la multiplication

du virus. Dans des conditions optimales, les symptômes et la multiplication du virus sont éliminés totalement.

Comme exemple de fragment d'acide nucléotidique préféré, on citera un fragment de l'extrémité 5' de l'ARN2 génomique du BNYVV ou du cDNA correspondant, codant pour au moins une partie de la protéine codée par les nucléotides 145 à 2218 du ARN2 ou pour une variante de cette protéine présentant une homologie d'au moins 80 % et comportant l'insertion, la substitution ou la délétion d'acide(s) aminé(s) et qui confère une résistance à la rhizomanie aux cellules l'exprimant.

Un autre exemple d'une plante transgénique selon cet aspect de l'invention est celle dans laquelle ledit fragment code pour la protéine codée par les nucléotides 145 à 708 et, en outre, pour une partie de la protéine codée par les nucléotides 709 à 2218 de l'ARN2 du BNYVV. Une telle plante transgénique peut comporter, selon l'invention, un fragment qui code pour la protéine codée par les nucléotides 145 à 871 de l'ARN2 du BNYVV, ce fragment pouvant être composé, par exemple, par les nucléotides 91 à 871 de l'ARN2 du BNYVV.

Le fragment transformant peut aussi coder pour au moins une partie d'une variante de la protéine codée par les nucléotides 145 à 2218, ladite variante se distinguant de la séquence sauvage par la présence de la séquence Glu Asp Leu Pro qui remplace les acides aminés His ALa codés par les nucléotides 253 à 258 de la séquence sauvage. Par séquence sauvage, il faut comprendre la séquence de l'ARN2 publiée par Bouzoubaa et al (1986), en particulier celle de la figure 2 de ladite publication. Cette figure indique la séquence nucléotidique ainsi que la séquence d'acides aminés.

La numérotation des bases utilisée dans cette demande est la même que celle appliquée par Bouzoubaa et al.

Comme exemples de parties de ce type de variante, on peut citer un fragment qui est composé par les nucléotides 91 à 871 de la séquence sauvage, les nucléotides 253 à 258 de la séquence sauvage étant remplacés par ceux codant pour Glu Asp Leu Pro. La partie de la variante peut aussi correspondre à celle codée par les nucléotides 145 à 255 dans la séquence sauvage, les nucléotides 253 à 255 de la séquence sauvage étant remplacés par ceux codant pour Glu.

Encore un exemple d'une plante transgénique selon cet aspect de l'invention est celle dans laquelle ledit fragment code pour au moins une partie d'une variante de la protéine codée par les nucléotides 145 à 2218, ladite variante se distinguant de la séquence sauvage par la présence de la séquence Arg Ser Ser Gly au lieu des acides aminés codés par les nucléotides 637 à 651 de la séquence sauvage, la séquence Arg Ser Ser Gly formant le carboxy-terminal de la protéine.

Plus particulièrement, les plantes transgéniques, selon ces aspects de l'invention, expriment des fragments d'acide nucléique consistant en les nucléotides 91 à 871 du ARN2 du BNYVV ou de la séquence cADN correspondante, dans lequel les nucléotides 253 à 258 sont éventuellement remplacés par GAA GAT CTT CCT, ou dans lequel la séquence GA AGA TCT TC a été insérée à la position 638, immédiatement après le C en position 637, ou encore les fragments BglII de ces séquences.

Selon un autre mode de réalisation de l'invention, le fragment transformant peut consister en les nucléotides 2078 à 2774 du ARN2 subgénomique du BNYVV. La protéine ainsi exprimée correspond au NH_2 terminal de la protéine de 42kDa du BNYVV.

Des exemples de fragments d'acide nucléique transformants particulièrement préférés et les protéines codées par ces fragments sont illustrés dans le tableau 1 (voir exemple 6).

Parmi ces séquences particulièrement préférées, on citera la séquence de bases consécutives codant pour au moins une partie de la protéine de capside de 22 Kd (codée par les nucléotides 145 à 708) et, en outre, pour une partie de la protéine de 75 Kd (codée par les nucléotides 709 à 2218). Un exemple d'une telle séquence est celle composée des nucléotides 91 à 871 du ARN2. Ce type de séquence incluant le codon stop du 22 Kd donne lieu au phénomène de "readthrough" et la cellule exprime ainsi deux protéines à la fois.

L'invention concerne également les protéines produites par l'expression de ces séquences, et en particulier la protéine de 29 Kd résultant de l'expression des nucléotides 91 à 871 du ARN2, qui est exprimée en même temps que la protéine de capside de 22 Kd.

Les promoteurs qui peuvent être utilisés dans les plantes transgéniques résistantes à la rhizomanie sont tous ceux qui permettent l'expression de la séquence conférant la résistance dans la plante. Les promoteurs 35S et Nos qui sont, respectivement le promoteur du grand transcrit 35S du virus de la mosaïque du choux-fleur et le promoteur du gène de la nopaline synthase sont particulièrement préférés. L'utilisation de ces promoteurs constitutifs, et en particulier le p35S, donne lieu d'une manière surprenante à une expression spécifique de la protéine protectrice dans les racines.

D'autres signaux de transcription à utiliser avec le promoteur sont des terminateurs, par exemple Nos.

Les graines des plantes transgéniques résistantes à la rhizomanie font également partie de cet aspect de l'invention.

Les séquences utilisées dans la transformation des plantes de l'invention peuvent être utilisées comme sonde nucléique, en combinaison avec des moyens permettant une détection de l'hybridation, pour détecter la présence des séquences dans des cellules transformées. Ceci peut être utile pour vérifier l'état transformé des cellules.

Cet aspect de l'invention concerne également un procédé de production de plantes transgéniques appartenant à l'espèce Beta vulgaris et résistante à l'infection par le BNYVV, ladite plante exprimant, spécifiquement dans les racines, une protéine capable de conférer ladite résistance, ledit procédé comprenant la transformation, par l'intermédiaire d'Agrobacterium tumefaciens, de cellules provenant de Beta vulgaris avec un des fragments tels que décrits ci-dessus, la transcription dudit fragment étant sous le contrôle d'un promoteur constitutif tel que le p35S ou le pNos, suivi de la régénération d'une plante transgénique à partir des cellules transformées.

Les différents aspects de l'invention seront illustrés par les exemples non limitatifs suivants. Ces exemples illustrent notamment :

- la production de fragments de message génétique modifié ou non du virus du BNYVV, par la plante,
- l'expression de ces fragments d'ADN d'origine virale sous des promoteurs constitutifs (35S ; Nos),
- la vérification de cette expression par la mise en évidence des ARN messagers correspondants dans des suspensions cellulaires habituées et transformées,

- la confirmation de la production dans la même cellule de deux protéines de tailles différentes codées par le même gène :

* la protéine de capside du BNYVV de 22 Kd (188, acides aminés)

* la protéine chimérique dérivée de la protéine de capside, de 29 Kd (252 acides aminés),

- l'utilisation des trois souches désarmées d'Agrobacterium tumefaciens pour la transformation de suspensions cellulaires et de cals friables de betterave,

- l'obtention de suspensions cellulaires transformantes, induites à partir de cals sélectionnés sur kanamycine après transformation de suspensions cellulaires habituées,

- l'utilisation de ces suspensions cellulaires transformées comme modèle pour tester l'expression de tout fragment d'ADN placé sous le contrôle de promoteurs appropriés,

- l'utilisation de ces suspensions cellulaires pour tester l'inhibition de la multiplication virale par un gène donné,

- l'inhibition de la multiplication du BNYVV dans des protoplastes issus de suspensions cellulaires transformées et produisant les deux protéines de 22 Kd et 29 Kd, des protoplastes servant comme modèle pour tester l'infectabilité de la cellule, les cellules ne pouvant pas être infectées directement par les virus,

- la transformation de jeunes cals friables organogènes,

- l'obtention de cals transformés organogènes sélectionnés sur kanamycine après transformation de jeunes cals friables organogènes,

- la possibilité de supprimer très tôt à la fois l'agent sélectif (kanamycine) et l'agent

bactériostatique (céfotaxime) du milieu de culture des cals,

- le développement des bourgeons ou embryons initiés à partir des cals transformés, en plantes transformées enracinées,

- l'expression dans la plante des gènes transférés,

- la mise en évidence de la production des protéines 22 et 29 Kd spécifiquement dans les racines de betteraves transformées,

- l'absence de la production de protéines 22 et 29 Kd dans la partie aérienne des plantes transformées,

- la production de graines transgéniques à partir de transformants primaires,

- la mise en évidence de la résistance à la rhizomanie des plantules issues des graines transgéniques.

Les figures 1 à 11 et les tableaux 1 à 3 illustrent différents aspects de l'invention, notamment :

Figure 1 :

Organisation génétique de l'ARN2 du BNYVV d'après Bouzoubaa et al, (1986)

Figure 2 : construction des vecteurs d'expression pBIOS1 et pBIOS3.

pBIOS1 contient le promoteur (pNos) et le terminateur (Nos 3') du gène de la nopaline synthétase séparés par un site de restriction BamHI (B) et encadrés par deux sites EcoRI (E).

pBIOS3 est un dérivé de pBIOS1 où le promoteur Nos a été remplacé par le promoteur du transcrit 35S (p35S) du Cauliflower Mosaic Virus (CaMV) isolé du plasmide pJEA25 (don de T. Michael).

Abréviations : B, BamHI - E, EcoRI - H, Hind III - Hp, HphI - P, Pst I - S, SmaI - Sst, SstI - X, XhoII - T4 DNA pol, T4 DNA polymérase - K, klenow polymérase - E Met, EcoRI Méthylase - Ap, Ampicilline - bp, paire de base.

Figure 3 : gènes chimériques codant pour la protéine de capside du BNYVV et pour la protéine chimérique dérivée.

A partir du plasmide pBC2 contenant l'ADN copie de l'extrémité 5' du RNA2, nous avons isolé un fragment DraI-Bgl I de 780 bp (position 91 à 871). Ce fragment a été traité par la T4 DNA polymérase et des linkers BamHI ont été rajoutés permettant son clonage au site BamHI des vecteurs d'expression pBIOS1 et pBIOS3. Les deux plasmides pBIO1-1B et pBIO3-1B obtenus ont le fragment d'ADN en orientation sens.

Abréviation : B, BamHI - D, Dra I - E, EcoRI - P, PstI - Sst, Sst 1 - T4 DNA Pol, T4 DNA Polymérase - CAP, départ théorique du transcrit - A+, signal de polyadénylation - ATG, codon de départ - TGA, TAA, codons stop.

Figure 4 : construction de pBIO1-2B et pBIO1-5B.

Figure 5 : construction des vecteurs de transfert.

Les différentes entités génétiques fonctionnelles dérivées de pBIOS1 et de pBIOS3 sont insérées dans le vecteur binaire pGA492. Les dérivés de pBIOS1 sont clonés au site EcoRI et seuls sont retenus les construits orientés dans le sens de transcription identique au gène de la néomycine phophotransférase (npt), alors que les dérivés de pBIOS3 sont insérés entre le site SstI et le site EcoRI, et se trouvent obligatoirement dans la bonne orientation. 32 plasmides dérivés de pGA492 ont donc été obtenus.

Abréviations : E, EcoRI - Sst, SstI - npt, néomycine phosphotransférase - cat, chloroamphénicol acétyltransférase - BR et BL, bordures droite et gauche du T-DNA - Tet, tétracycline résistance - Kb, kilobase.

Figure 6 : vecteur binaire pGA-β -3-1B.

Légende : 35S, promoteur 35S du Cauliflower Mosaic Virus - 5'Nos, promoteur du gène de la nopaline synthase - Nos, terminateur du gène de la nopaline synthase. NPT, gène de la néomycine phosphotransférase -UID A, gène de la -glucuronidase d'E. coli - BR et BL, bordures droite et gauche du T-DNA de pTiT 37 - amp, gène de résistance à l'ampicilline - tet., gène de résistance à la tetracycline - Kana., gène de résistance à la kanamycine - E, EcoRI - H, Hind III - S, Sst I - ● ,origine de réplication du RK2.

Figure 7 : analyse par Western blot des suspensions cellulaires transformées par le vecteur binaire pGA- β-3-1B.

Légende : CP, protéine de capside - WT, suspension non transformée - 20 ng et 2 ng, reconstructions avec les quantités indiquées de BNYVV β-Glu, β-glucuronidase - MW, marqueur de poids moléculaire.

En encadré, la structure du gène chimérique codant pour la protéine de capside est représenté. p35S, promoteur du Cauliflower Mosaic Virus - Nos, terminateur du gène de la nopaline synthase ; tag, codon de terminaison du BNYVV : readthrough - tag, codon de terminaison situé dans le terminateur - atg, codon d'initiation.

Figure 8 : infection de protoplastes de betterave par du BNYVV F13 (□ ,O) et S2 (▽) par la méthode au PEG. (▲) représente les résultats obtenus avec du virus inactivé.

Figure 9 : infection de protoplastes de betterave par électroporation en présence (▼) et en absence (△) de CaCl2 5 mM. Les symboles ouverts correspondants représentent le pourcentage de protoplastes viables en présence (▽) et en absence (□) de CaCl2 5 mM.

Figure 10 : profils densitométriques d'analyses de RNA extraits de protoplastes infectés isolés de lignées exprimant (----) ou n'exprimant pas (——) la protéine de capside du BNYVV.

Figure 11 : analyse par Western blot d'extraits protéiques de 3 betteraves transgéniques à l'aide de deux sérums : un sérum anti-BNYVV et un sérum anti-β -glucuronidase.

Abréviations : L, limbe ; P, pétiole ; R, racine ; Te, témoin ; S, suspension cellulaire transformées ; 2ng, 2ng de BNYVV purifié ; 14-3, 22-1 et 68-1 réfèrent à 3 betteraves transgéniques différentes.

Tableau 1 : les 16 gènes de résistance potentiels au BNYVV avec leurs produits théoriques.

N.B. : les chiffres entre parenthèses réfèrent la position des sites de restrictions selon la séquence du BNYVV ; Δ représente le linker BglII additionné. La taille des transcripts correspond à la partie du messager complémentaire du BNYVV.

Tableau 2 : infection par le BNYVV de protoplastes issus de cellules transformées et non transformées. 1 x 10^6 protoplastes ont été inoculés avec 5 µg de BNYVV (F13 ou S2) sauf pour l'expérience 4 où l'inoculum consistait de 30 µg de BNYVV les chiffres représentent le pourcentage de protoplastes fluorescents détectés 30 h après l'inoculation. CP+ et CP- sont des lignées cellulaires transformées exprimant et n'exprimant pas la protéine capsidaire du BNYVV.

Tableau 3 : différents milieux de culture utilisés dans le procédé de transformation et de régénération selon l'invention.

EXEMPLE 1 : EXTRACTION DE L'ARN VIRAL

La multiplication du virus BNYVV se fait sur Chenopodium quinoa après inoculation manuelle, et l'extraction se fait à partir de feuilles virosées de huit jours selon la technique de Putz (1977). La suspension virale ainsi obtenue est ajustée à 100 mM NaCl puis soumise à deux extractions au phénol suivies de deux lavages à l'éther de la phase aqueuse. Les RNA viraux sont précipités par addition de deux volumes d'éthanol distillé et conservés à -20°C. Le nombre et la taille des RNA a été rapporté par Richards et al (1985).

EXEMPLE 2 : FABRICATION DU cDNA DU RNA 2

Richards et al ont montré en 1985 que le RNA 2 était un messager efficace pour la synthèse de la protéine capsidaire. Un ADN complémentaire d'une partie du RNA 2 a été synthétisé par la méthode de Van der Werf et al (1981) et cloné au site Pst 1 du plasmide pBR322 (Bolivar et al, 1977). Le plasmide résultant pBC2 contient un ADN copie correspondant aux 2938 bases de l'extrémité 5' du RNA 2 (Richards et al, 1985).

EXEMPLE 3 : DETERMINATION DE LA SEQUENCE DE L'EXTREMITE 5'DU RNA 2

Grâce à la technique de Maxam et Gilbert (1980), et à celle de Sanger (1977), la séquence du clone pBC2 a été déterminée. La séquence détaillée est présentée dans la publication de Bouzoubaa et al (1986) avec l'organisation génétique du RNA2 schématisée. L'examen de cette séquence a confirmé la présence de la protéine de capside au niveau du 1er cistron situé en 5', le codon ATG de départ étant placé à 144

nucléotides de l'extrémité 5'. Cette protéine d'un poids moléculaire de 22 Kd comporte 188 codons et se termine par un codon ambre UAG. La composition en acides aminés déduite de la séquence est identique à celle de la protéine capsidaire du BNYVV déterminée par C. Putz (1977). Le codon stop (UAG) est immédiatement suivi d'une phase ouverte ayant une capacité codante correspondant à un polypeptide de 54 Kd. Par la suppression du codon UAG, on peut donc obtenir une phase de lecture ouverte capable de coder pour une protéine de 75 Kd. Ces données sont en accord avec les résultats obtenus par Ziegler et al (1985) montrant que le RNA2 est un messager efficace pour la synthèse de deux protéines immunoprécipitées par du sérum anti BNYVV et dont la synthèse est augmentée en présence d'un t.RNA suppresseur. Tous ces résultats sont discutés dans la publication de Bouzoubaa et al (1986), il est aussi précisé qu'une 3ème phase de lecture capable de coder in vitro pour une protéine se fait à partir d'un ARN subgénomique du RNA2 qui a été mis en évidence et qui peut être encapsidé.

EXEMPLE 4 : CONSTRUCTION DES VECTEURS D'EXPRESSION

Premièrement les signaux de transcription du gène de la nopaline synthase de la bactérie Agrobacterium tumefaciens T 37 ont été utilisés. A partir du plasmide pNopnéoΔ 18 construit par Bevan (1983) dont la structure est présentée dans la figure 2, celui-ci contient la séquence du vecteur de clonage pUC9 (Vierra et al, 1982) avec un fragment EcoRI-BamHI de 260 bp et un fragment HindIII-XhoII (BamHI-BglI) de 310 bp. Ces deux fragments qui proviennent du plasmide tumorigène pTiT37 d'Agrobacterium tumefaciens T 37 contiennent respectivement le signal de polyadénylation et le promoteur du gène de la nopaline

synthase. Ces deux signaux de transcription encadrent un fragment BglII-Bam HI de 1000 bp qui contient le gène codant pour l'aminoglycoside phosphotransférase II ; à partir de ce plasmide, le fragment de 1000 bp a été enlevé par digestion ménagée avec l'enzyme de restriction XhoII, et les molécules de taille de 3300 bp environ sont récupérées après migration sur gel d'agarose. Après ligation et transformation dans E. coli HB101 (Bolivar et al, 1979) des transformants résistants à l'ampicilline ont été sélectionnés. La majorité de ces techniques sont décrites dans "Molecular Cloning. A laboratory manual" (Maniatis et al, 1982). Grâce à la cartographie à l'aide d'enzyme de restriction, les inventeurs ont retenu le plasmide pNosΔNéo, qui possédait un fragment EcoRI-BamHI de 260 bp et un fragment BamHI-HindIII de 310 bp. Pour des commodités d'utilisation de ce plasmide, les inventeurs ont remplacé le site de restriction Hind III par un site EcoRI (détails non présentés) et ainsi ils ont obtenu le vecteur d'expression pBIOS1 (figure 2).

Un autre vecteur d'expression a été construit dans le cadre de l'invention. Il a la même structure que pBIOS1, le même fragment comportant le signal de polyadénylation, mais le fragment promoteur a été changé. En effet, le fragment PstI-BamHI comportant le promoteur du gène de la nopaline synthase a été remplacé par un fragment EcoRI-BamHI de 400 bp contenant le promoteur du grand transcrit 35S du virus de la mosaïque du choufleur (Ca.M.V.). Ce fragment a été obtenu à partir du plasmide pUC35S qui dérivait du clonage d'un fragment BamHI-HphI au site Sma I du vecteur de clonage pUC13 (Messing, 1983). La source du fragment BamHI-HphI était le plasmide pJEA25 construit par T. Michael qui avait prélevé un fragment Dde I

s'étendant de la position 7069 à la position 7569 du CaMV isolat BJ1 décrit par Franck et al (1980). Après avoir modifié les extrémités de ce fragment grâce à des linkers BamHI, T. Michael l'avait cloné au site BamHI du plasmide pAT153 (Twigg et al, 1980). Sur la figure 2, les différentes étapes de la construction du vecteur d'expression pBIOS3 sont schématisées.

Pour les deux vecteurs d'expression pBIOS1 et pBIOS3, le site de restriction BamHI est présent entre les deux fragments d'ADN contenant le promoteur pour l'un et pour l'autre le signal de polyadénylation ; cette situation est idéale pour l'insertion de tout DNA étranger qui sera un substrat pour la transcription.

EXEMPLE 5 : INSERTION DE FRAGMENTS cDNA DU BNYVV AU SITE BAMHI DES VECTEURS D'EXPRESSION

Le plasmide pBC2 a été digéré par les enzymes de restriction BglI et DraI et ensuite l'extrémité sortante du site BglI a été supprimée par l'utilisation de la DNA polymérase du bactériophage T4. Ce fragment a été purifié par élution à partir d'un gel d'agarose, après séparation électrophorétique. Des linkers BamHI ont été additionnés aux extrémités de ce fragment, et après une digestion avec un grand excès de l'enzyme de restriction BamHI ; ce fragment de 780 bp a été incubé avec les vecteurs pBIOS1 et pBIOS3 ouvert au site BamHI. Après ligation, le mélange a servi à transformer E. coli HB101. Après un tri effectué sur les clones résistants à l'ampicilline, 4 plasmides ont été retenus. Les plasmide appelés pBIO1-1B et pBIO3-1B qui ont le fragment cDNA contenant la séquence codante de la protéine de capside sous le contrôle du promoteur Nos pour le premier et pour l'autre sous le contrôle du 35S (figure 3).

Ces plasmides peuvent diriger la fabrication de RNA messagers qui une fois traduits, produiront à la fois la protéine de capside du BNYVV et une protéine chimérique de 29 Kd provenant de la suppression du codon stop (phénomène de readthrough). Cette protéine de 252 acides aminés est composée comme suit :

- les 188 acides aminés de la protéine de capside,
- 53 acides aminés de la protéine de 75Kd allant de la position 189 à 241 inclus,
- de 11 acides aminés codés par la séquence du terminateur de la nopaline synthase qui sont respectivement : thréonine, glycine, serine, proline, isoleucine, leucine, glutamine, thréonine, phénylalanine, glycine, glutamine. Deux autres plasmides ont été obtenus, ces plasmides appelés pBIO1-1A et pBIO3-1A ont le fragment cDNA du BNYVV en orientation inverse sous le contrôle des deux promoteurs. Ces plasmides pourront diriger la fabrication de RNA messagers dits antisens, qui seront des messagers complémentaires de la partie 5' du RNA2.

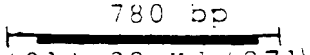
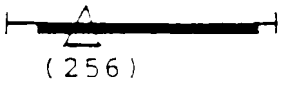
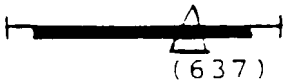
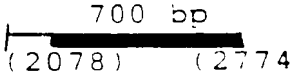
EXEMPLE 6 : CONSTRUCTION D'UNE FAMILLE DE GENES PERMETTANT LA FABRICATION DE PROTEINES DE CAPSIDE DU BNYVV MODIFIEES ET DE RNA ANTISENS DE TAILLE VARIABLE

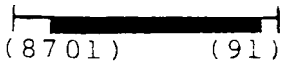
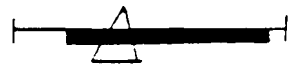
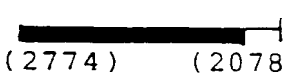
Deux plasmides dérivés de pBIO1-1B, le plasmide pBIO1-2B et le plasmide pBIO1-5B ont été construits. Les différentes étapes de la construction de ces plasmides sont présentées dans la figure 4. Brièvement pour la construction de pBIO1-2B, le plasmide pBIO1-1B a été ouvert au site SphI (position 256 du RNA2) les bouts "collants" ont été enlevés par l'utilisation de la DNA polymérase du bactériophage T4 (délétion de 4 bp) et des linkers BglII de 10 bp ont été ligasés aux extrémités franches ainsi produites. Après une digestion exhaustive avec BglII, le plasmide est

religasé. Après transformation dans E. coli, le plasmide pBIO1-2B a été obtenu ; celui-ci ne contient plus de site SphI et à cette position on trouve maintenant un site BglII. Cette manipulation a permis deux choses : premièrement, l'obtention d'un site de restriction utile pour d'autres constructions (extrémités compatibles avec celles produites par BamHI) et deuxièmement, l'obtention d'une séquence codante modifiée qui code pour une protéine de capside possédant deux acides aminés supplémentaires et dont deux acides aminés sont changés. Le plasmide pBI01-5B a été obtenu par le même type de manipulation mais dans son cas c'est le site SmaI de pBIO1-1B (position 637 du RNA2) qui a été modifié par des linkers BglII. Ce plasmide code pour une protéine de capside raccourcie de 19 acides aminés et dont les 4 derniers sont modifiés.

En utilisant les sites BglII et Bam HI de ces deux plasmides, les inventeurs ont pu isoler et cloner dans les deux vecteurs d'expression pBIOS1 et pBIOS3 différents morceaux de cDNA recouvrant l'extrémité 3' ou l'extrémité 5' de la protéine de capside et ceci sur des longueurs variables. Une famille de gènes susceptibles de rendre résistante au BNYVV une cellule ou une plante fabriquant le produit d'un ou plusieurs de ces gènes a ainsi été obtenue. Tous ces différents fragments de cDNA sont présentés dans le tableau 1 avec les produits attendus (transcrit et protéine) ; la lettre B référant une orientation sens et A une orientation antisens.

TABLEAU 1

CODE	FRAGMENT cDNA	TRANSCRIPT	PROTEINE
1B		ARN bon sens 780 bp	protéine de capside de 22 Kd (188 acides aminés) et protéine chimérique dérivée 29 Kd (252 Aa)
2B		ARN bon sens 780 bp	Protéine de capside mutée (190 Aa)
3B		ARN bon sens 3' 165 bp	36 Aa NH2 terminal de la protéine de capside
4B		ARN bon sens 5' 615 bp	
5B		ARN bon sens 780 bp	Protéine de capside tronquée (169 Aa)
6B		ARN bon sens 5' 546 bp	164 Aa NH2 terminal de la protéine de capside
7B		ARN bon sens 3' 234 bp	
8B		ARN bon sens 700 bp	215 Aa NH2 terminal de la protéine de 42 Kd

CODE	FRAGMENT cDNA	TRANSCRIPT	PROTEINE
1 A	 (8701) (91)	ARN anti-sens 780 bp	
2 A		ARN anti-sens 780 bp	
3 A		ARN anti-sens 5' 165 bp	
4 A		ARN anti-sens 3' 615 bp	
5 A		ARN anti-sens 780 bp	
6 A		ARN anti-sens 5' 546 bp	
7 A		ARN anti-sens 3' 234 bp	
8 A	 (2774) (2078)	ARN anti-sens 700 bp	

N.B. : les chiffres entre parenthèses repèrent la position des sites de restrictions selon la séquence du BNYVV ; ∇ représente le linker BglII additionné. La taille des transcripts correspond à la partie du messager complémentaire du BNYVV.

Sur ce tableau, il est aussi présenté un fragment de cDNA s'étendant de la position 2078 à 2774 ; il s'agit d'un fragment Sau 3A du RNA2 correspondant à l'extrémité 5'du RNA subgénomique codant pour la protéine de 42 Kd (figure 1b). Les inventeurs ont cloné ce fragment dans les vecteurs d'expression pBIOS1 et pBIOS3 et ceci dans les deux orientations (8B et 8A).

EXEMPLE 7 : INTRODUCTION DES GENES DANS UN VECTEUR BINAIRE

Le vecteur binaire pGA492 choisi a été construit par G. An (1986).Ce plasmide a une taille de 12 kb, sa carte génétique est présentée dans la figure 5. Ce plasmide possède :

- deux fragments d'ADN contenant les séquences du T-DNA du plasmide tumorigène pTiT37, ces séquences délimitent la partie transférée et sont indispensables au transfert,
- un gène chimérique contenant la séquence codante du gène de la néomycine phosphotransférase du transposon Tn5 (Rothstein et al, 1981) qui est fusionné à un fragment contenant le promoteur du gène de la nopaline synthase et les premiers codons de la séquence codante de ce même gène. Ce gène chimérique est terminé par le signal de polyadenylation du gène de la nopaline synthase. Ce gène confère aux cellules végétales le contenant la capacité de se multiplier en présence de kanamycine, qui est normalement un antibiotique toxique pour celles-ci,
- plusieurs sites uniques de restriction permettant le clonage de gènes construits in vitro,
- une origine de réplication à large spectre d'hôte fonctionnant dans E. coli et dans Agrobacterium tumefaciens,
- une origine de transfert et les gènes mob nécessaires pour le transfert par conjugaison bactérienne,
- un gène de résistance à la tétracycline qui permet la sélection de bactéries transconjugates,
- un gène de résistance à la tétracycline qui permet la sélection de bactéries transconjugantes.

Sur la figure 5, les inventeurs ont exemplifié l'introduction de deux gènes, les gènes portés par

pBIO1-1B et par pBIO3-1B. Tous les fragments de cDNA sous le contrôle du promoteur Nos (pBIOS1 dérivés) sont clonés au site EcoRI de pGA 492 après isolement des gènes respectifs avec EcoRI. Seuls les clones présentant les gènes dans le même sens de transcription que celui du gène conférant la résistance à la kanamycine sont retenus. Pour les gènes contenant le promoteur 35S du CaMV (pBIOS3 dérivés) le clonage s'effectue entre les sites Sst I et EcoRI de pGA 492 après digestion des diférents plasmides par ces deux mêmes enzymes. 32 vecteurs de tranfert ont été obtenus, deux de ceux-ci sont présentés dans la figure 5, pGA-1-1B et pGA-3-1B.

EXEMPLE 8 : INTRODUCTION D'UN GENE REPORTEUR DANS LE VECTEUR BINAIRE pGA-3-1B

La confirmation du caractère transformé d'un tissu végétal s'effectue dans de nombreux cas par la mise en évidence des protéines codées par les gènes transférés. Dans le cas du vecteur binaire pGA-3-1B, les inventeurs ont pu doser par des techniques immunologiques la protéine de capside du BNYVV et par un dosage enzymatique l'activité de la néomycine phosphotransférase qui confère la résistance à la kanamycine. Cependant, ces dosages sont longs et délicats et ils nécessitent une importante quantité de matériel végétal.

En 1987, Jefferson et al, utilisent le gène uid A d'E. coli codant pour l'enzyme β-glucuronidase (GUS E.C.3.2.2.31) comme gène reporteur. Ce gène sous le contrôle de signaux de transcription végétaux (promoteur 35S du CaMV et terminateur Nos) s'exprime très bien dans les cellules végétales et l'enzyme se révèle très stable. Plusieurs méthodes de dosage de l'activité enzymatique de cette protéine sont disponibles car il existe différents types de

substrats ; en particulier des substrats donnant des produits chromogènes et des substrats donnant des produits fluorescents. On peut effectuer de façon simple et avec peu de matériel végétal des dosages quantitatifs (intensité de la fluorescence) et des dosages qualitatifs par histochimie (apparition d'un précipité bleu-indigo). Pour bénéficier de ce système reporteur dans les expériences de transformation, les inventeurs ont construit le vecteur binaire pGA-β-3-1B. La structure génique de ce plasmide est présentée dans la figure 6. Le gène pouvant inhiber la multiplication du BNYVV est encadré par le gène conférant la résistance à la kanamycine et par le gène codant pour la β-glucuronidase. Pour réaliser cette construction, les inventeurs ont inséré au site EcoRI du vecteur binaire pGA-3-1B, le plasmide pBI221 (Jefferson, 1987) ouvert à sont site EcoRI. Le vecteur binaire pGA-β-3-1B résultant (d'une taille de 19 kb) a été transféré dans les souches d'Agrobacterium désarmées (LBA 4404, EHA 101, C58'3) par conjugaison triparentale selon la technique décrite par Ditta et al (1980).

EXEMPLE 9 : OBTENTION DE CALS FRIABLES REGENERANTS A PARTIR DE FEUILLES

Des graines de betterave sont semées dans du terreau en serre. Ces graines sont issues de la variété américaine "REL 1".

Environ un mois après la germination en serre, les premières expériences d'induction de cals selon la méthode décrite par Saunders et al (1986) ont été faites :

- de jeunes feuilles de 3 à 5 cm de long sont prélevées de chaque plante,
- elles sont désinfectées comme suit :

détergent de la marque Domestos 15 % pendant 5 minutes

3 rinçages à l'eau stérile

séchage sur papier filtre stérile

- chaque feuille est ensuite découpée en fragments de 0.25 cm^2 environ,

- les explants ainsi obtenus sont mis en culture sur du milieu MSB1 (Tableau 3) en boîtes de Pétri,

- les boîtes après avoir été scellées avec un film plastique de la marque Scello-frais sont placées à 30°C à l'obscurité pendant 30 jours,

- puis elles sont sorties en chambre de culture L:D 18:8, 25°C:20°C.

De 4 à 10 semaines après la mise en culture, des cals blancs friables apparaissent autour, sur ou sous les explants foliaires.

De 1 à 8 semaines après l'apparition de cals des bourgeons et/ou embryons commencent à régénérer de ces cals.

TABLEAU 3 : MILIEU DE CULTURE

MS :

Pour 1 litre :

4.4 g de milieu de Murashige et Skoog déshydratés de chez SIGMA réf. M6899.

Vitamines :

1 mg panthoténate de calcium

0,01 mg biotine

1 mg acide nicotinique

1 mg pyridoxine HCl

10 mg thiamine HCl

30 g saccharose

pH : 5.8

Milieu solide : 8 g agar-agar

MSB1 :
MS + 1 mg/l BAP

MSBo.1 :
MS + 0.1 mg/l BAP

MS enracinement :
MS + 1 mg/l ANA
Etalement des cellules :
MSB1 + C = MSB + 300 mg/l céfotaxime
MSB1 + CK = MSB + 300 mg/l céfotaxime + 200 mg/l kanamycine

LB :
Pour 1 litre :
10 g bactotryptone
5 g yeast extract
10 g NaCl

EXEMPLE 10 : OBTENTION DE SUSPENSIONS CELLULAIRES A PARTIR DES CALS INDUITS

De 4 à 6 semaines après leur apparition, les cals sont prélevés (en évitant toute structure organisée) et mis en culture dans 100 ml de milieu MSB1 liquide, dans les erlenmeyers de 250 ml fermés avec une feuille de cellophane maintenue au col par deux élastiques. Les erlenmeyers sont agités à 200 RPM environ dans la chambre de culture.

La suspension cellulaire s'établit en 2 ou 3 semaines. Chaque suspension est repiquée toutes les 3 semaines environ comme suit :

- le contenu de chaque erlenmeyer après 3 semaines de culture est filtré sur une série de trois tamis empilés (dont les mailles sont de 1 mm, 500 µm et 100 µm). Une partie de chaque fraction (>1 mm, >500µm, >100 µm) est remise en suspension dans 100 ml

de milieu MSB1 frais en erlenmeyers de 250 ml. Ces nouvelles suspensions sont à nouveau agitées à 200 RPM. L'observation des différentes suspensions cellulaires établies à partir des différents génotypes ont permis de distinguer deux types cellulaires :

* type habitué (A) : suspension fine, verte à croissance rapide, régénère ponctuellement des formations vitrifiées se développant difficilement,

* type noduleux (C) : suspension d'agrégats compacts jaunâtres, à croissance plus lente, régénère plus fréquemment que la première, des structures embryonnaires compactes se développant assez bien.

EXEMPLE 11 : TRANSFORMATION DE SUSPENSIONS CELLULAIRES ET DE JEUNES CALS

Suspension cellulaire

La transformation est faite sur des suspensions cellulaires après 3 semaines de culture, sans repiquage.

Dans un tube plastique, stérile et gradué, on recueille une partie de la suspension de manière à avoir 5 ml de cellules tassées dans 10 ml de milieu. 10 ml milieu MSB1 frais sont rajoutés. La nouvelle suspension ainsi obtenue est distribuée dans quatre boîtes de pétri à raison de 5 ml par boîte.

Les souches d'Agrobacterium tumefaciens testées sont LBA 4404, EHA 101, C58'3. Chacune de ces souches contient des vecteurs binaires portant les gènes construits (exemples 5 et 6) et plus particulièrement le vecteur pGA-β-3-1B (exemple 8).

Les souches bactériennes sont conservées à -20°C dans 15 % de glycérol. 50 μl de chaque souche sont prélevés et mis en culture dans 2 ml de milieu LB + rifampicine + tetracycline. Les cultures sont agitées à 200 RPM à 30°C pendant 2 jours. Chaque souche est

repiquée dans du milieu frais et cultivée dans les conditions décrites plus haut pendant une nuit.

Quand les bactéries sont ainsi prêtes, l'infection des cellules végétales est faite :

- 50 µl de chaque souche poussée une nuit sont prélevés et ajoutés à une des boîtes de Pétri contenant les cellules de betteraves décrites plus haut

- la coculture des cellules de betterave et des bactéries se fait à l'obscurité pendant 3 jours en chambre de culture

Après ces 3 jours, des cellules végétales sont lavées pour éliminer la bactérie, une première fois avec du MSB1 + 600 mg/l de céfotaxime (bactériostatique inhibant la croissance d'Agrobacterium), puis une deuxième fois dans du milieu MSB1 + 300 mg de céfotaxime

- les cellules ainsi lavées sont mises en culture sur un disque de papier Whatman stérile déposé sur du milieu MSB1 + CK (tableau 3), en boîtes de Pétri (la kanamycine est l'agent sélectif permettant aux seules cellules transformées de se développer). Les boîtes sont scellées avec du scello-frais et mises en chambre de culture. 15 jours après, les filtres portant les cellules végétales sont repiqués sur du milieu frais MSB1 + céfotaxime + kanamycine

- 3 à 8 semaines après la coculture, des cals blancs apparaissent sur un lit de cellules mortes.

Quand ils sont suffisamment développés, ces cals sont repiqués soit sur milieu MSB1 + CK ou MSB1 + C ; la plupart des cals poussent sur les deux milieux. Un test histochimique (Jefferson, 1987) pour déceler l'activité de la protéine codée par le gène de la β-glucuronidase dans les cellules de ces cals, a permis d'évaluer à environ 80 % le taux de cals positifs pour

ce test. Ceci confirme donc le caractère transgénique de la plupart des cals obtenus.

Le fait de cultiver ces cals sans l'agent sélectif (kanamycine) ne semble pas modifier l'expression du gène GUS. De plus, après un mois de culture sur milieu avec céfotaxime, les cals peuvent être sevrés de cet antibiotique sans que la bactérie se développe sur le milieu.

Donc, un mois après le clonage des cals, on peut se passer d'ajouter les deux antibiotiques dans le milieu de culture, sans inconvénient apparent. Ceci peut représenter un atout pour la régénération.

Dispersion de cals nouvellement induits

Au lieu de transformer des suspensions cellulaires induites depuis plusieurs mois (et ayant hypothétiquement perdu de leur potentiel de régénération), de jeunes cals fraîchement induits d'explants foliaires (Saunders et al, 1986) étaient transformés. Le potentiel organogène de ces cals transformés pouvait ainsi être investigué.

Pour tester cela, des cals juste apparus depuis 2 à 6 semaines sur feuilles de serre ont été prélevés. Ces cals ont été dispersés dans un milieu MSB1 liquide en tubes plastiques stériles, et le même protocole de transformation que pour les suspensions cellulaires a été appliqué. Des cals transformés ont ainsi été sélectionnés par cette voie.

EXEMPLE 12 : EXPRESSION DES GENES POUVANT INHIBER LE DEVELOPPEMENT DU BNYVV DANS DES CELLULES DE BETTERAVE

Les inventeurs ont transformé des suspensions cellulaires habituées de betteraves avec tous les gènes construits présentés dans les exemples 5 et 6. Pour chacun de ces gènes plusieurs suspensions cellulaires transformées ont été initiées et cultivées

en présence de kanamycine 200 mg/l. A partir de 10 g de cellules transformées soumises à l'action de cellulases et de pectinases, soit les ARN totaux, soit les protéines solubles totales ont été isolés.

Pour l'extraction des ARNs la technique utilisant l'isothyocyanate de guanidium décrite par Ausubel et al (1987) s'est révélée la plus efficace sur ces cellules aux parois digérées. Les ARN totaux ont été analysés par Northern blot selon Maniatis et al (1982). Pour l'hybridation des ARN totaux contenant un ARN message dérivé du gène de la protéine de capside, les inventeurs ont utilisé comme sonde le fragment BamHI de 780 paires de bases du plasmide pBIO-3B. Pour ceux exprimant un ARN messager dérivé du gène codant pour la protéine de 42 Kd, le fragment EcoRI de 1330 paires de bases du plasmide pBIO-3-8B a été utilisé comme sonde. Le marquage radioactif de ces 2 sondes grâce à de l'ATP (P32) s'est fait par la technique dite de l'"Oligonucleotide Primed Synthesis" (Ausubel et al, 1987).

Les résultats obtenus ont permis aux inventeurs de vérifier la présence de tous les ARN messagers attendus, ce qui montrait la fonctionnalité des gènes chimériques construits. Ces messagers ont une taille correspondante à celle indiquée dans le tableau 1 majorée de 200 nucléotides environ. Ces nucléotides supplémentaires situés à l'extrémité 3' du mRNA sont dus à la partie transcrite du terminateur Nos jusqu'au signal de polyadénylation et à la queue de poly-A. Il a aussi été observé que le promoteur Nos a une efficacité de transcription de 30 à 50 fois plus faible que le promoteur 35S dans des cellules de betteraves. Ces résultats sont comparables à ceux obtenus sur le tabac et la tomate par Sanders et al (1987).

Bien que la transcription de tous les gènes ait été vérifiée, il était nécessaire de voir si la traduction et la production de la protéine de capside et de la protéine chimérique de 29 Kd a été obtenu dans le cas du vecteur binaire pGA-β-3-1B. Pour cela il a été réalisé un western blot (Ausubel et al, 1987)) sur des protéines solubles extraites à partir de différentes suspensions cellulaires transformées (tampon d'extraction : urée, 9M ; β-mercaptoéthanol, 7,5 %, SDS, 4,5 % ; pH : 6,8). Les résultats sont présentés dans la figure 7 ; la partie supérieure du filtre a été révélée avec des anticorps polyclonaux anti β-glucuronidase et la partie inférieure par des anticorps polyclonaux anti-BNYVV. La β-glucuronidase est présente dans la majorité des suspensions transformées (bande immunoréagissante 68 Kd). Dix suspensions sur les 11 testées présentent une bande immunoréactive commigrant avec la protéine de capside du BNYVV ainsi qu'une bande d'un poids moléculaire d'environ 29 Kd. L'expression de ces deux protéines est de l'ordre de 0,005 à 0;01 % des protéines solubles totales ; cette quantité atteste de la bonne efficacité de traduction du messager produit. Il est à noter que la quantité de 29 Kd est particulièrement élevée dans la majorité des transformants et peut être supérieure à celui de la protéine de capside. Le taux de readthrough dans les suspensions cellulaires habituées semble être supérieur à celui observé dans des racines de betteraves pour le RNA2 (Ziegler et al, 1985) et sur des betteraves transgéniques (exemple 15).

EXEMPLE 13 : TEST DE RESISTANCE IN VITRO

Isolement et purification de protoplastes de betterave

Cinq jours après le repiquage, 2 ml de suspension cellulaire tassée sont digérés dans 20 ml d'enzymes caylase. La composition du cocktail enzymatique est la suivante : 0.25 %, 345 S ; 0.25 % T ; 0.08 % M_2L dissout dans du mannitol 0.7 M contenant 0.08 mM NaH2PO4, 0.03 mM MES et 0.68 mM CaCL2. La pression osmotique est ajustée à 760 mOsmole/Kg et à pH 5,8. après 16 à 18H00 de douce agitation (30 oscillations par minute) à l'obscurité et à 24°C, la suspension est filtrée sur tamis de 100 et 50 μm respectivement. Le filtrat est additionné d'un volume égal d'une solution iso-osmotique de KCl à 470 mM et les protoplastes sont sédimentés à 50 g pendant 5 minutes. Le culot est repris dans du saccharose isoosmotique à 570 mM et centrifugé à 50 g pendant 15 minutes. L'anneau de protoplastes est prélevé, soumis à une deuxième centrifugation dans du saccharose, qui, sédimenté, est lavé deux fois dans du mannitol à 760 mOsmoles/Kg. La viabilité des protoplastes est quantifiée par coloration au diacétate de fluorescéine (Widholm, 1972). On s'assure que la digestion est complète par l'absence de coloration de paroi du calcofluor.

L'utilisation de gradients iso-osmotiques mais de densités différentielles a permis d'obtenir des préparations de protoplastes à très hauts rendements 5 x 10^6 protoplastes par ml de cellules tassées et complètement débarrasés de débris cellulaires. En moyenne, 90 % des protoplastes sont viables.

Culture des protoplastes isolés de cellules non transformées et transformées

Le milieu de culture des protoplastes est un milieu de macro et micro éléments de Murashige et Skoog (1962) où le NH_4NO_3 est diminué de moitié et additionné de :

1g/l hydrolysat de caséine
30 g/l saccharose
7,7 mg/l glycine
1,3 mg/l d'acide nicotinique
0,25 ng/l pyridoxine
0,25 n/l Thiamin-HCI
400 mg/l glutamine
25 mg/l glucosamine
1 mg/l 6 benzyl amino purine
1 mg/l acide indole acétique.

La pression osmotique est ajustée à 760 mOsmole/kg avec du mannitol et le pH à 5,8. Le milieu de culture est stérilisé par filtration.

Des protoplastes ont été isolés à partir de suspensions cellulaires transformées et non transformées. Ils ont été mis en culture dans le milieu ci-dessus en présence et en absence de kanamycine à 200 mg/l afin de calculer l'efficacité d'étalement. Les résultats démontrent que les protoplastes isolés à partir de cellules non transformées ne survivent pas sur milieu sélectif contenant 200 mg/l de kanamycine. Ceci est en accord avec l'absence d'échappement dans les expériences de transformation. Aucune différence significative n'a été trouvée entre les efficacités d'étalement de protoplastes issus de cellules transformées en présence et absence de kanamycine. Ceci confirme donc l'absence de cellules non transformées au sein des lignées transformées. Lors d'expériences comparatives

qui seront décrites ci-dessous, les protoplastes issus de 4 types de lignées cellulaires (β7 et β14 exprimant la 22 Kd et la 29 Kd, WT, non transformées et β D transformée par le vecteur binaire pGA492) seront mis en culture en absence de pression de sélection.

Infection de protoplastes de betterave par le BNYVV

L'optimisation de la technique d'infection s'est effectuée sur des protoplastes issus de cellules non transformées. Les inventeurs se sont inspirés de la technique d'infection par le polyéthylène glycol décrit par Samac et al (1983) et de celle par électroporation décrite par Watts et al (1987). Afin d'obtenir des taux élevés d'infectivité, il était imperatif d'utiliser des préparations de protoplastes dépourvues de débris, ayant des taux de viabilité supérieurs à 90 % ainsi que des préparations de virus âgées de moins de deux semaines.

La fréquence d'infection a été déterminée par immunofluorescence par une technique indirecte décrite par Maule et al (1980). Les protoplastes infectés ont une fluorescence vert clair caractéristique, dispersée dans le cytoplasme ; les protoplastes non infectés sont marron terne. La figure 8 présente les résultats de 4 expériences d'infection de protoplastes de betterave par la technique au polyéthylène glycol. Il a été constaté qu'après 24 heures de culture environ 60% des protoplastes sont infectés. Ce pourcentage n'évolue pas de façon significative pour des durées de culture supérieures à 24 h. L'infection s'est donc réalisée de façon quasi-synchrone. Cette figure montre aussi que du virus inactivé par congélations et décongélations répétées ne se réplique pas dans les protoplastes de betterave. Dans toutes les

expérimentations effectuées, en moyenne 50 % des protoplastes sont infectés.

Les inventeurs ont réussi à introduire des particules virales du BNYVV en utilisant des impulsions électriques (technique d'électroporation). Les inventeurs se sont inspirés de la technique décrite par Watts et al (1987) et ont utilisé un électroporateur à décharge de capacités (Guerche et al, 1987).

L'optimisation de la technique s'est faite en faisant varier la durée d'impulsion délivrée par des condensateurs de différentes capacités et la résistivité du milieu d'électroporation. C'est ainsi que les inventeurs ont vu que deux impulsions de 100 ms délivrées à 15 secs d'intervalle par des condensateurs de capacité 63 μF chargés à 220 V à 1 x 10^6 protoplastes remis dans du mannitol contenant du CaCl2 à 5 mM en présence de 5 μg de BNYVV permettaient d'avoir un taux de 55 % de protoplastes infectés. L'omission du CaCl2 résulte en une diminution de l'infectivité d'un facteur 3, bien que la viabilité des protoplastes soit augmentée d'un facteur 2. Ceci est illustré sur la figure 9.

Infection de protoplastes de betteraves issus de cellules transformées et non transformées

Afin de déterminer si la protéine capsidaire (22 kD) du BNYVV et la protéine dérivée (29kD) produite dans les cellules de betterave pouvait inhiber la multiplication du virus, les inventeurs ont inoculé des protoplastes issus de cellules exprimant ou n'exprimant pas ces protéines par le virus. Les expériences ont été réalisées par les techniques au PEG et par l'électroporation. L'infection a été visualisée par immunofluorescence des cellules 30 heures après culture. Il n'a pas été décelé de bruit

de fond en immunofluorescence dans les cellules transformees et exprimant la protéine capsidaire avant inoculation.

TABLEAU 2 : infection par le BNYVV de protoplastes issus de cellules transformées et non transformées.

	Source protoplastes				
	Non transformé		Transformé CP$^+$	Transformé CP$^-$	Protection (%)
Expérience	P11	β7	β14	βD	
1	30		1		97
2	68		10		85
3	70		4		94
4	27		1		96
5	35	5			86
6	25	2			92
7	37	10		36	73
8		12		48	75

1×10^6 protoplastes ont été inoculés avec 5 µg de BNYVV (F13 ou S2) sauf pour l'expérience 4 où l'inoculum consistait de 30 µg de BNYVV les chiffres représentent le pourcentage de protoplastes fluorescents détectés 30 h après l'inoculation. CP+ et CP- sont des lignées cellulaires transformées exprimant et n'exprimant pas la protéine capsidaire du BNYVV.

Le tableau 2 résume les résultats obtenus avec deux lignées cellulaires (β7 et β14) exprimant fortement deux protéines, et la lignée cellulaire transformée (βD) et la lignée cellulaire non transformée WT. Dans tous les cas, le pourcentage de protoplastes infectés

isolés de cellules transformées β7 et β14 est significativement plus faible que celui des protoplastes de cellules non transformées. Les expériences 7 et 8 prouvent bien que la protection est bien due à la présence des protéines 22 Kd et 29 Kd, car le taux d'infection de protoplastes issus des cellules de la lignée βD est comparable à celle des cellules non transformées. Donc le processus de transformation en lui-même ne confère pas de protection à l'infection par le virus. L'expression de protéines étrangères, autres que celles du BNYVV ne confère pas non plus la protection à l'infection par ce même virus. La protection n'est pas abolie lorsque l'on augmente la concentration du virus présente dans l'inoculum. Les profils densitométriques (figure 10) des RNA totaux (isolés de protoplastes β14 et WT infectés) et hybridés avec une sonde cDNA total contre les 4 RNA de BNYVV montrent que ceux-là ne se répliquent pas dans les protoplastes exprimant la protéine capsidaire du virus. La protection a aussi été observée lorsque l'inoculation a été effectuée par électroporation indiquant que la protection est indépendante de la méthode utilisée pour l'inoculation.

Ces résultats démontrent pour la première fois que des protoplastes de betterave exprimant la protéine capsidaire virale du BNYVV et la protéine chimérique dérivée sont protégés contre l'infection par ce même virus.

EXEMPLE 14 : REGENERATION DE PLANTES A PARTIR DE CALS TRANFORMES

Après un premier passage d'un mois sur M.SB1 + C ou MSB1 + CK, les cals tranformés sont repiqués tous les mois sur MSB1. Après des délais plus ou moins longs, variant d'une semaine à plusieurs mois, des

bourgeons et/ou embryons régénèrent sur certains de ces cals.

Il a été observé qu'après transformation les cals ont le même phénotype que la suspension de départ. C'est-à-dire que le type noduleux ou habitué se retrouve après la transformation. En outre, il semble que ce soit les cals tranformés de type noduleux qui aient la plus grande aptitude à la régénération. Ils permettent la régénération de plusieurs structures se développant assez bien et vite en plante, alors que les structures obtenues sur les cals habitués transformés sont très rares, longues et très difficiles à se développer en plante.

Les structures régénérées sont très hétérogènes morphologiquement. Elles sont repiquées, après avoir été coupées à la base au scalpel, sur le milieu MSB0.1 en boîtes de Pétri.

Quand les bourgeons commencent à développer plusieurs feuilles, ils sont remis en multiplication végétative en pots ou boîtes. Les bourgeons les plus développés sont alors mis en enracinement sur MS + ANA 1 mg/l(ANA : acide naphtalène-acétic). Les racines apparaissent de 2 à 6 semaines après.

Dès que les racines sont suffisamment développées, les plantes sont acclimatées en serre dans du terreau universel. Au bout de 3 mois, les plantes sont bien développées (voir photo) et ont perdu la plupart des variations phénotypiques dues à la culture in vitro.

EXEMPLE 15 : EXPRESSION DE LA PROTEINE DE CAPSIDE ET DE LA PROTEINE CHIMERIQUE DERIVEE DANS LES BETTERAVES TRANSGENIQUES

Bien que sachant les betteraves transgéniques, grâce aux tests de détection du gène codant pour la β-glucuronidase, il n'était pas certain que le gène

intéressant, à savoir celui codant pour les protéines de capside du BNYVV, était bien exprimé dans ces plantes. Pour le savoir, il faut détecter ces protéines dans les tissus de betterave transformées. Pour détecter les protéines dans les transformants, il a été fait appel à la technique de Western blot (Ausubel et al, 1987). Celle-ci consiste à faire migrer les protéines solubles extraites de broyat de tissus des transformants, sur gel de polyacrylamide en conditions dénaturantes (Laemmli, 1970). Les protéines ainsi séparées sont tranférées par électrotransfert sur une membrane de nitrocellulose. Cette membrane est ensuite hybridée avec des anticorps polyclonaux de lapins dirigés contre le BNYVV. La révélation se fait par addition d'anticorps anti-lapin conjugés à la phosphatase alcaline, qui utilise des substrats chromogènes. La figure 11 montre le type de résultats obtenus. Les extraits de betterave transgéniques contenant le gène codant pour les protéines de capside montrent une bande immunoréactive migrant au même niveau que la protéine de capside du virus. Cette bande n'est pas présente dans les extraits de plante non transformées.De plus, dans les extraits de plantes transformées, on détecte une bande à 29 Kd correspondant à la protéine chimérique dérivée de la 22 Kd par addition de 64 acides aminés, due au "readthrough" (exemple 5). Ces deux protéines de 22 et 29 Kd n'ont pu être détectées que dans des extraits de racines de transformants et pas dans les feuilles. La figure 11 illustre donc l'expression spécifique, dans les racines de betteraves transgéniques, des protéines de 22kD et de 29kD, malgré l'utilisation d'un promoteur constitutif. La fonctionnalité du promoteur dans les parties aériennes de la plante est confirmée

par l'expression du gène marqueur GUS dans tous les tissus.

Le BNYVV se transmet et se développe au niveau des racines, il est donc avantageux que les protéines susceptibles d'inhiber le virus soient présentes dans ces organes.

EXEMPLE 16 : OBTENTION DE GRAINES DE BETTERAVES TRANSGENIQUES

Après 2 à 3 mois d'acclimatation en serre des plantes transgéniques, il a été constaté qu'elles étaient phénotypiquement conformes à la plante mère. A ce stade les plantes possèdent de 10 à 15 feuilles très bien développées, et sont toujours à l'état de rosette. Pour savoir si les plantes obtenues sont tout à fait normales et notamment fertiles, et si le gène introduit est transmis à la descendance, les plantes ont été vernalisées pour induire la montée à graine. Les transformants primaires sont donc placés entre 2 et 7°C à l'obscurité pendant 3 mois, puis en champs en période de jour long. Un mois à un mois et demi après la mise en champ, la hampe florale commence à monter.

La floraison a lieu trois mois environ après la sortie de vernalisation, et les fruits sont mûrs deux mois après.

Quand les glomérules sont bien secs, ils sont récoltés et nettoyés. Les graines sont ainsi prêtes à être semées.

EXEMPLE 17 : APPLICATION DE LA METHODE DE L'INVENTION A DIFFERENTES VARIETES DE BETA VULGARIS :

Les méthodes de transformation et de régénération décrites dans les exemples 9 à 14 ont été appliquées à des suspensions cellulaires et à des dispersions de cals blancs friables issues de plantes provenant d'une variété "élite" (variété parente d'hybrides commerciaux).

Des plantes transgéniques exprimant la protéine de capside du BNYVV ont été obtenues. Cette expression était spécifique dans les racines. La variété "élite" ayant un environnement génétique différent des autres variétés transformées, l'expression spécifique semble être alors conservée dans l'espèce Beta vulgaris.

La reproductibilité de ces techniques a été vérifiée en appliquant les méthodes de transformation et de régénération de l'invention à d'autres variétés et en d'autres sites géographiques par une autre équipe d'expérimentateurs. Dans chaque cas, des plantes transgéniques ont été obtenues.

EXEMPLE 18 : AUTOFECONDATIONS ET CROISEMENTS DES PLANTES TRANSGENIQUES RESISTANTES A LA RHIZOMANIE

Des graines transgéniques ont été obtenues à la fois sur des autofécondations des plantes transformées et sur des croisements de ces mêmes plantes avec trois autres lignées de betteraves mâles stériles (une lignée mâle stérile génique monogerme, une lignée mâle stérile génique multigerme et une lignée mâle stérile cytoplasmique).

L'expression spécifique de la protéine de capside et de celle de la protéine de 29kD dans les racines de toutes les plantes transgéniques issues d'autofécondations et de croisements a été obtenue.

Ces résultats attestent que cette expression spécifique est maintenue dans un environnement génétique différent de celui des transformants primaires.

BIBLIOGRAPHIE

ABEL P.P., NELSON R.S., DE B., HOFFMANN N., ROGERS S.G., FRALEY R.T., BEACHY R.N. (1986) - Science 232 : 738-743.

AKIYOSHI D.E., MORRIS R.O., HINZ R., MISCHKE B.S.; KOSUGE T., GARFINKEL D.J., GORDON M.P., NESTER E.W. (1983) - PNAS 80 : 407-411.

AN G. (1985) - Plant Physiol. 79 : 568-570.

AN G., WATSON B.D., STACHEL S., GORDON M.P., NESTER E.W. (1985) - EMBO J. 4 : 277-284.

AN G. (1986) - Plant Physiol. 81 : 86-91.

AUSUBEL F.M., BRENT R., KINGTON R.E., MOORE D.D., SMITH J.A., SEIDMAN J.G. and STRUHL K. (1987) - Current Protocol in Molecular Biology - Published by Green Publishing Associates and Wiley Interscience.

BEVAN M., FLAVELL R.B., CHILTON M.D. (1983) - Nature 304 : 184-187.

BEDBROOK J.R., CHALEFF R.S., FALCO S.C., MAZUR B.J., YADAV N.S., (1988) - EP-A-0257993

BEVAN M. (1984) - Nucl. ac. Res. 12 : 8711-8721.

BOLIVAR F.; RODRIGUEZ R.L., GREEN P.I., BETLACH M.C., HEYNECKER H.L., BOYER H.W., CROSA J.H., FALKOW S. (1977) - Gene 2 : 95-113.

BOLIVAR F., BACKMAN K. (1979) - Methods in Enzymol. 68 : 245-267.

BOUZOUBAA S., ZIEGLER V., BECK D., GUILLEY H., RICHARDS K., JONARD G. (1986) - J. Gen. Virol. 67 : 1689-1700.

BROADBENT L. (1976) - Ann. Rev. Phytopathol. 14 : 75.

CUOZZO M., O'CONNEL K.M., KANIEWSKI W., FANG R.X., CHUA N.H. and TUMER N.E. (1988) - Biotechnology 6 : 549-557.

DALE P.J., MARKS M.S., BROWN M.M., WOOLSTON C.J., GUNN H.V., MULLINEAUX P.M., LEWIS D.M., KEMP J.M., CHEN

D.F., GILMOUR D.M. and FLAVELL R.B. (1989) - Plant Science 63 : 237-245.

DE BLOCK M., BOTTERMAN J., VANDEWIELE M., DOCKX J., THOEN C., GOSSELE V., RAO MOVVA N., THOMSON C., VAN MONTAGU M., LEEMANS J., (1987) EMBO J. 6 : n° 9, 2513-18.

DETREZ C. TETU T., SANGWAN R.S., SANGWAN-NORREEL B.S. (1988) - J. Exp. of Bot. 39 (204) : 917-926.

DITTA G., STANFIELD S., CORBIN D., HELINSKI D.R. (1980) - PNASS 77 : 7347-7351.

FERNOW K.H. (1967) - Phytopathol. 57 : 13-47.

FISCHOFF D.A., et al (1987), BIO/TECHNOLOGY 5 : 807-813.

FRANCK A., GUILLEY H., JONARD G., RICHARDS K., HIRTH L. (1980) - Cell 21 : 285-294.

FREYTAG A.H., ANAND S.C., RAO-ARELLI A.P. and OWENS L.D. (1988) - Plant Cell Reports 7 : 30-34.

GERLACH W.L., LLEWELLYN D. and HASELOFF J. (1987) - Nature 328 : 802-805.

GIELEN J.J.L., et al , Abstracts of 7th International Congress of Plant Tissue and Cell Culture. I.A.P.T.C. Juin 1990, Amsterdam.

GUERCHE P., BELLINI C., LE MOULLEC J.M., CABOCHE M. (1987) - biochimie 69 : 621-628.

HARRISSON D.B., MAYO M.A. and BAULCOMBE (1987) - Nature 328 : 799-802.

HARPSTER M.H., TOWNSEND J.A., JONES J.D.J., BEDBROOK J., DUNSMUIR P.(1988) - Mol. Gen. Genet. 212 : 182-190.

HOEKEMA A., HIRSCH P.R., HOYKAAS P.J.J. and SCHILLPEROOT R.A. (1983) - Nature 303 : 179-180.

HOOD E.E., HELMER G.L., FRALEY R.T., CHILTON M.D. (1986) - J. Bact. 168 : 1291-1301.

JEFFERSON R.A., KAVANAGH T.A. and BEVAN M.W. (1987) - EMBO J. 6 : 3901-3907.

KRENS F.A., ZIJLSTRA C., VAN DEN MOLEN W., JAMAR D. and HUIZING H.J. (1988) - Euphytica S. 185 : 194.

LAEMMLI V. (1970) - Nature 277 : 680-685.

LEWELLEN R.T., SKOYEN I.O. and ERICHSEN A.W. (1987) - in 50th Winter Congress I.I.R.B. Bruxelles 11-12 February 1987, 136-156.

LINDSEY K. and JONES M.G.K. (1989) - Plant Cell Reports 8(2) : 71-74.

LOESH-FRIES L.S., MERLO D., ZIMMENT T., BURHOP L., HILL K., KROHN K., JARNIS N., NELSON S., HALK E. (1987) - EMBO J. 6 : 1845-1851.

MANIATIS T., FRITSCH E.F., SAMBROOK J. (1982) - Molecular cloning. Cold Spring Harbor Laboratory.

MARIANI C., et al (1990), NATURE, vol. 347 : 737-741.

MAULE A.J., BOULTON M.I., WOOD K.R. (1980) - Phytopath Z. 97 : 118-126.

MAXAM A.M. and GILBERT W. (1980) - Methods Enzymol., 65 : 499-560.

MESSING J. (1983) - in methods in Enzymology 101 : 20-78 Academic Press, New York.

MURASHIGE T., SKOOG F. (1962) - Plant Physiol 15 : 473-497.

PERLAK J., et al, (1990) BIO/TECHNOLOGY, vol. 8, 939-943.

PUTZ C. (1977) - J. Gen. Virol. 35 : 397-401.

PUTZ C., RICHARD-MOLARD M. (1984) - C.R. Acad. Agr. de France 70 : 370-378.

RICHARDS K., JONARD G., GUILLEY H., ZIEGLER V. and PUTZ C. (1985) - J. Gen. Virol. 66 : 345-350.

RITCHIE G.A., SHORT K.C., DAVEY M.R. (1989) - J. Exp. Bot. 40(211) : 277-283.

ROTHSTEIN S.J., JORGENSEN R.A., YIN J.C.P., YONG-DI Z., JOHNSON R.C., REZNIKOFF W.S. (1980) - Cold Spring Harbor Symp. Quant. Biol 45 : 99-106.

SALLE G., LE GOZ S. and TOQUET C. (1986) - Physiol. Veg. 24 : 73-83.

SAMAC D.A., NELSON S.E., LOESCH-FRIES L.S. (1983) - Virology 131 : 455-462.

SANDERS P.R., WINTER J.A., BARNASSON A.R., ROGERS S.G., FRALEY R.T. (1987) - Nucl. Acid Res. 15 : 1543-1548.

SANGER F., NICKLEN S., COULSON A.R. (1977) - PNAS 74 : 5463-5467.

SAUNDERS J.W. and DOLEY W.P. (1986) - J. Plant. Physiol. 124 : 473-479.

SCOTT R.J. and DRAPER J. (1987) - Plant Mol. Biol. 8 : 264-274.

TAMADA T. and BABA T. (1973) - Ann. Phytopath. Soc. Japan 39 : 325-332.

TETU T., SANGWAN R.S. and SANGWANN-NORREEL B.S. (1988) - J. Exp. Bot, 38 (188) : 506-517.

TUMER N.E., O'CONNEL K.H., NELSON R.S., BEACHY R.N., FRALEY R.T., SHAH D.H. (1987) - EMBO J. 6 : 1181-1188.

TWIGG A.J. and SHERATT D. (1980) - Nature 283 : 216-218.

VAECK M, et al (1987), NATURE, 328, 33-37.

VAN DER WERF S., BREGEGERE F., KOPECKA H., KITAMURA N., ROTHBERG P.G., KOURILOSKY P., WIMMER E., GIRARD M. (1981) - PNAS 78 / 5983-5987.

VAN DUM M.P., BOL J., VAN VLOTEN-DOTING L. (1987) - Virology 159 : 299-305.

VIEIRA J., MESSING J. (1982) - Gene 19 : 259-268.

WATTS J.W., KING J.M., STACEY N.J. (1987) - Virology 157 : 40-46.

WIDHOLM J.M. (1972) - Stain Tech. 47 : 189-194.

YACOUB A. and TEPPER D. (1987) - in 50th Winter Congress I.I.R.B. Bruxelles 11-12 February 1987, 107-308.

ZIEGLER V., RICHARDS K., GUILLEY H., JONARD G., PUTZ C. (1985) - J. Gen. Virol. 66 : 2079-2087.

REVENDICATIONS

1. Procédé de transformation de cellules végétales appartenant à l'espèce Beta vulgaris caractérisé en ce qu'il comprend la mise en contact d'une dispersion de cals blancs friables dans un milieu de culture cellulaire végétale liquide contenant 0 à environ 3.0 mgL^{-1} d'une cytokine, ou d'une suspension de cals blancs friables dans un milieu de culture cellulaire végétale liquide contenant environ 0.1 à environ 3.0 mgL^{-1} d'une cytokinine, avec Agrobacterium contenant un vecteur portant un gène destiné à être introduit dans les cellules végétales, suivie de coculture des cellules végétales et des bactéries pour donner lieu à des cals friables transformés.

2. Procédé selon la revendication 1, caractérisé en ce qu'il comprend les étapes successives suivantes :

I) induction de cals blancs friables à partir d'explant ;

II) dispersion des cals dans un milieu de culture cellulaire végétale liquide contenant 0 à environ 3.0 $mgl-^{1}$ d'une cytokinine, ou obtention d'une suspension cellulaire à partir des cals dans un milieu de culture cellulaire végétale liquide contenant environ 0.1 à environ 3.0 $mgl-^{1}$ d'une cytokinine ;

III) mise en contact de la dispersion ou de la suspension cellulaire, avec Agrobacterium tumefaciens contenant un vecteur portant un gène destiné à être introduit dans les cellules végétales, suivie de coculture des cellules végétales et des bactéries ;

IV) lavage des cellules végétales pour éliminer les bactéries et sélection des cellules transformées sur un milieu sélectif ;

V) culture des cellules transformées sélectionnées pour obtenir des cals friables transformés.

3. Procédé selon l'une quelconque des revendications 1 ou 2, caractérisé en ce que les cals blancs friables sont induits à partir de jeunes feuilles, ayant par exemple une longueur de 3 à 5 cm, prelevées d'une plante âgée de moins de trois mois.

4. Procédé selon l'une quelconque des revendications 1 à 3 caractérisé en ce que la dispersion des cals s'effectue dans un milieu de culture cellulaire végétale contenant de la 6-benzylaminopurine (BAP), plus particulièrement environ $1mgl^{-1}$ BAP.

5. Procédé selon l'une quelconque des revendications 1 à 4, caractérisé en ce que la suspension cellulaire est obtenue par mise en culture pendant 2 à 3 semaines des cals blancs friables âgés de 4 à 6 semaines dans le milieu de culture additionné de cytokinine , le milieu étant agité pendant cette période, suivie de repiquage de la suspension ainsi obtenue sur du milieu de culture frais, donnant lieu à deux types cellulaires, notamment type habitué et type noduleux.

6. Procédé selon la revendication 5, caractérisé en ce que le milieu de culture est additionné de la 6-benzylaminopurine (BAP), plus particulièrement environ 1 mgl^{-1} BAP.

7. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que le milieu de culture cellulaire végétale est le milieu de Murashige et Skoog (1962), dit milieu M.S.

8. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que la coculture de cellules végétales et des bactéries s'effectue pendant 3 jours dans l'obscurité sur un

milieu de culture cellulaire végétale tel que le milieu MS, éventuellement additionné de cytokinine, par exemple environ 1 mgl^{-1} BAP.

9. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que l'élimination des bactéries s'effectue par lavage des cellules végétales avec un milieu de culture cellulaire végétale contenant un agent bactériostatique inhibant la croissance d'Agrobacterium, par exemple le céfotaxime.

10. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que la sélection des cellules transformées s'effectue sur un milieu contenant de la kanamycine.

11. Procédé selon l'une quelconque des revendications précédentes, caractérisé en ce que la culture des cellules transformées sélectionnées s'effectue sur un milieu de culture solide, tel que le milieu M.S solide, additionné de cytokinine, d'agent bactériostatique et d'agent sélectif.

12. Procédé selon l'une quelconque des revendications 1 à 11 caractérisée en ce que le gène destiné à être introduit dans les cellules végétales est choisi parmi un gène conférant un caractère d'intérêt agronomique ou industriel, par exemple un gène de résistance à l'infection par un virus, tel qu'un gène codant pour la protéine de capside du virus BWYV ou du virus BNYVV, un gène conférant une résistance à un herbicide ou à un insecticide, ou encore un gène dont l'expression confère la stérilité mâle

13. Procédé de régénération de bourgeons et/ou d'embryons transgéniques appartenant à l'espèce Beta vulgaris à partir d'explants, caractérisé en ce qu'il comprend les étapes successives suivantes :

I) obtention de cals friables transformés selon l'une quelconque des revendications 1 à 12 ;
II) repiquage des cals transformés sur un milieu de culture contenant 0 à environ 3 mgl^{-1} d'une cytokinine, et éventuellement un agent bactériostatique et un agent sélectif jusqu'à l'apparition de bourgeons et/ou d'embryons transgéniques.

14. Procédé selon la revendication 13, caractérisé en ce que le milieu de culture est le milieu M.S additionné d'environ 1mgl^{-1} BAP et éventuellement environ 300 mgl^{-1} cétotaxime et 200 mgl^{-1} kanamycine.

15. Procédé de régénération de plantes transgéniques appartenant à l'espèce Beta vulgaris, caractérisé en ce qu'il comprend les étapes successives suivantes :
I) régénération de bourgeons et/ou d'embryons transgéniques selon le procédé de la revendication 13 ;
II) repiquage des bourgeons et/ou des embryons transgéniques sur un milieu de culture tel que le milieu M.S additionné d'environ 0.1 mgl^{-1} cytokinine, par exemple de la BAP ;
III) remise des structures régénérées en multiplication végétative suivi d'enracinement sur un milieu de culture tel que le milieu M.S contenant de l'acide naphthalène acétique, par exemple environ 1 mgl^{-1}.

16. Procédé de production de graines de plantes transgéniques appartenant à l'espèce Beta vulgaris caractérisé en ce que qu'il comprend les étapes suivantes :
I) régénération de plantes transgéniques selon le procédé de la revendication 15 ;

II) vernalisation des plantes transgéniques et récolte des graines après floraison.

17. Cals friables transformés appartenant à l'espèce Beta vulgaris et pouvant être produits selon l'une quelconque des revendications 1 à 12.

18. Bourgeons et/ou embryons transgéniques appartenant à l'espèce Beta vulgaris et pouvant être produits selon l'une des revendications 13 et 14.

19. Plantes transgéniques appartenant à l'espèce Beta vulgaris et pouvant être produits selon le procédé de la revendication 15.

20. Graines de plantes transgéniques appartenant à l'espèce Beta vulgaris et pouvant être produits selon la revendication 16.

21. Plante transgénique appartenant à l'espèce Beta vulgaris et résistante à l'infection par le virus des nervures jaunes et nécrotiques de la betterave à sucre (BNYVV), ladite plante étant transformée d'une manière stable par un fragment d'acide nucléique, dont le produit d'expression est capable de conférer ladite résistance, ledit fragment étant dérivé de l'extrémité 5' de l'ARN2 génomique ou subgénomique du BNYVV, ou du cADN correspondant, ce fragment codant pour au moins une partie des protéines codées par les nucléotides 145 à 3285 de la séquence sauvage de l'ARN2, et étant sous le contrôle d'un promoteur permettant l'expression du fragment dans les cellules de la plante et étant dans l'orientation sens ou antisens.

22. Plante transgénique, selon la revendication 21, dans laquelle ledit fragment code pour au moins une partie de la protéine codée par les nucléotides 145 à 2218 de l'ARN2 ou pour une variante de cette protéine présentant une homologie d'au moins 80 % et comportant l'insertion, la substitution ou la délétion d'acide(s) aminé(s).

23. Plante transgénique, selon la revendication 22, dans laquelle ledit fragment code pour la protéine codée par les nucléotides 145 à 708 et, en outre, pour une partie de la protéine codée par les nucléotides 709 à 2218 de l'ARN2 du BNYVV.

24. Plante transgénique, selon la revendication 23, dans laquelle ledit fragment code pour la protéine codée par les nucléotides 145 à 871 de l'ARN2 du BNYVV.

25. Plante transgénique, selon la revendication 24, dans laquelle ledit fragment est composé par les nucléotides 91 à 871 de l'ARN2 du BNYVV.

26. Plante transgénique, selon la revendication 22, dans laquelle ledit fragment code pour au moins une partie d'une variante de la protéine codée par les nucléotides 145 à 2218, ladite variante se distinguant de la séquence sauvage par la présence de la séquence Glu Asp Leu Pro qui remplace les acides aminés His ALa codés par les nucléotides 253 à 258 de la séquence sauvage.

27. Plante transgénique selon la revendication 26, dans laquelle ledit fragment code pour une partie de la variante et est composé par les nucléotides 91 à 871 de la séquence sauvage, les nucléotides 253 à 258 de la séquence sauvage étant remplacés par ceux codant pour Glu Asp Leu Pro.

28. Plante transgénique, selon la revendication 26, dans laquelle ledit fragment code pour une partie de la variante, ladite partie correspondant à celle codée par les nucléotides 145 à 255 dans la séquence sauvage, les nucléotides 253 à 255 de la séquence sauvage étant remplacés par ceux codant pour Glu.

29. Plante transgénique, selon la revendication 26, dans laquelle ledit fragment code pour une partie de la variante, ladite partie correspondant à celle

codée par les nucléotides 256 à 871 dans la séquence sauvage, les nucléotides 256 à 258 de la séquence sauvage étant remplacés par ceux codant pour Asp Leu Pro.

30. Plante transgénique, selon la revendication 22, dans laquelle ledit fragment code pour au moins une partie d'une variante de la protéine codée par les nucléotides 145 à 2218, ladite variante se distinguant de la séquence sauvage par la présence de la séquence Arg Ser Ser Gly au lieu des acides aminés codés par les nucléotides 637 à 651 de la séquence sauvage, la séquence Arg Ser Ser Gly formant le carboxy-terminal de la protéine.

31. Plante transgénique, selon la revendication 30, dans laquelle ledit fragment code pour une partie de la variante et est composé par les nucléotides 91 à 871 de la séquence sauvage, les nucléotides 637 à 654 de la séquence sauvage étant remplacés par ceux codant pour Arg Ser Ser Gly stop.

32. Plante transgénique, selon la revendication 31, dans laquelle ledit fragment code pour une partie de la variante, ladite partie correspondant à celle codée par les nucléotides 144 à 640 de la séquence sauvage.

33. Plante transgénique, selon la revendication 31, dans laquelle ledit fragment code pour une partie de la variante et est composé par les nucléotides 641 à 871 de la séquence sauvage, les nucléotides 641 et 642 de la séquence sauvage étant remplacés par GA et les nucléotides 643 à 654 étant remplacés par ceux codant pour Ser Ser Gly Stop.

34. Plante transgénique, selon la revendication 21 comprenant un fragment de l'extrémité 5' de l'ARN2 subgénomique du BNYVV, ou du cADN correspondant, ledit fragment codant pour au moins une partie de la

protéine codée par les nucléotides 2133 à 3285 de l'ARN2, ou pour une variante de cette protéine présentant au moins 80 % d'homologie et comportant l'insertion, la substitution ou la délétion d'acide(s) aminé(s).

35. Plante transgénique, selon la revendication 34 dans laquelle ledit fragment code pour la protéine codée par les nucléotides 2133 à 2774 de l'ARN2 du BNYVV.

36. Plante transgénique, selon la revendication 35 dans laquelle ledit fragment est composé par les nucléotides 2078 à 2774 de l'ARN2 du BNYVV.

37. Plante transgénique, selon l'une quelconque des revendications précédentes, caractérisée en ce qu'elle exprime la protéine conférant la resistance, et codée par ledit fragment, uniquement dans les racines.

38. Plante transgénique, selon la revendication 37, caractérisée en ce que le promoteur contrôlant l'expression de ladite protéine est un promoteur constitutif, par exemple le pNos ou le p35S.

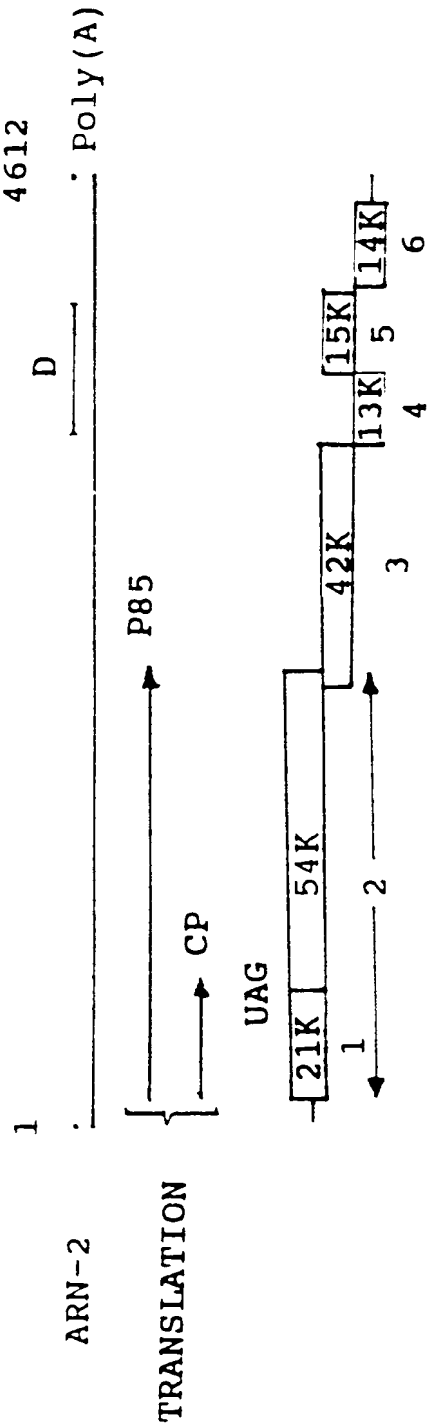
39. Plante transgénique selon la revendication 37, susceptible d'être obtenue par le procédé de la revendication 15.

40. Graines de plantes transgéniques selon l'une quelconque des revendications 21 à 39.

41. Procédé de production de plantes transgéniques appartenant à l'espèce Beta vulgaris et résistante à l'infection par le BNYVV, ladite plante exprimant, spécifiquement dans les racines, une protéine capable de conférer ladite résistance, ledit procédé comprenant la transformation, par l'intermédiaire d'Agrobacterium tumefaciens, de cellules provenant de Beta vulgaris avec un des fragments d'acide nucléique tels que décrits dans

l'une quelconque des revendications 1 à 16, la transcription dudit fragment étant sous le contrôle d'un promoteur constitutif tel que le p35S ou le pNos, suivi de la régénération d'une plante transgénique à partir des cellules transformées.

FIGURE 1


4612
Poly(A)
D
P85
CP
UAG
1
ARN-2
TRANSLATION
21K
54K
42K
13K
15K
14K
1
2
3
4
5
6

2/13

FIGURE 2

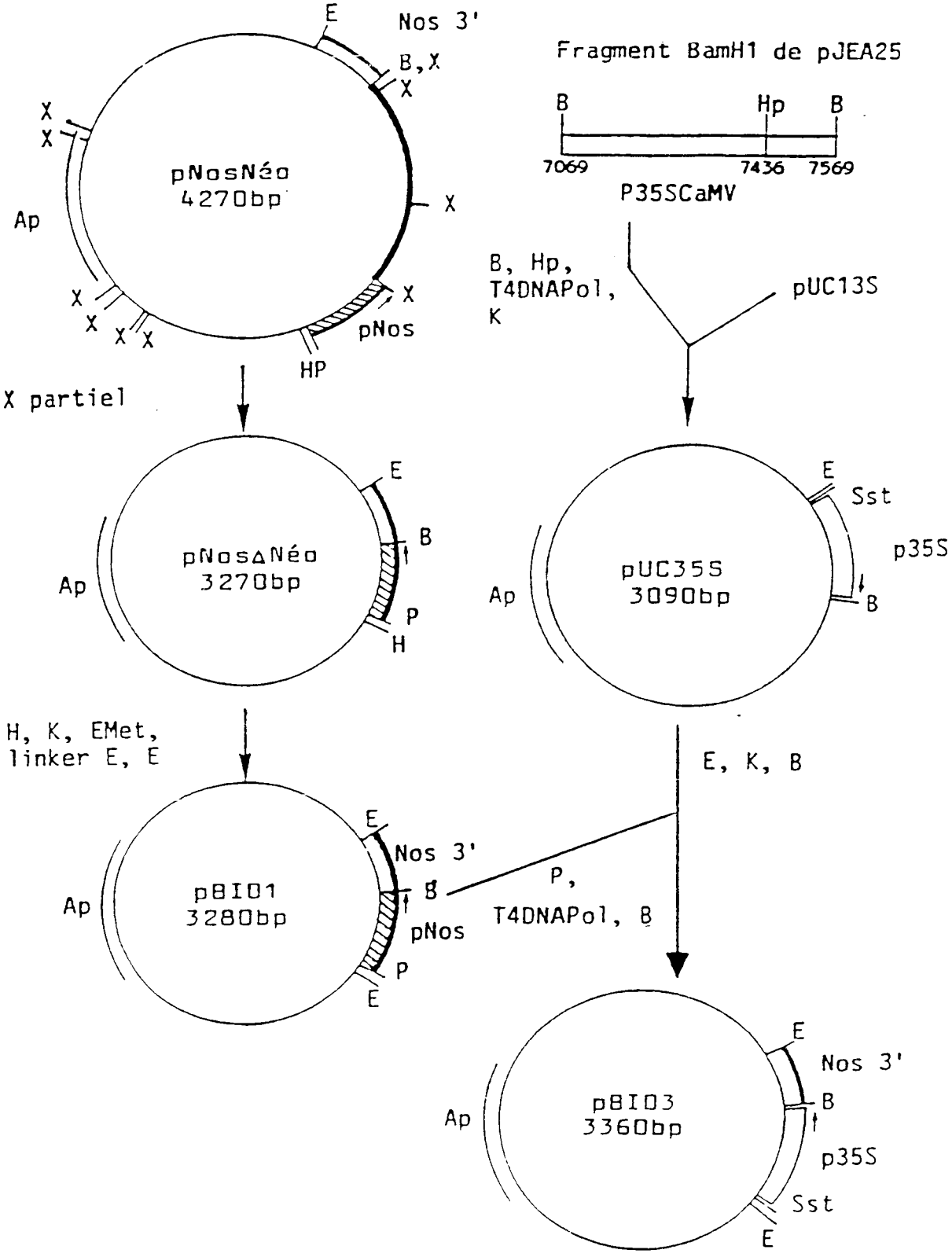
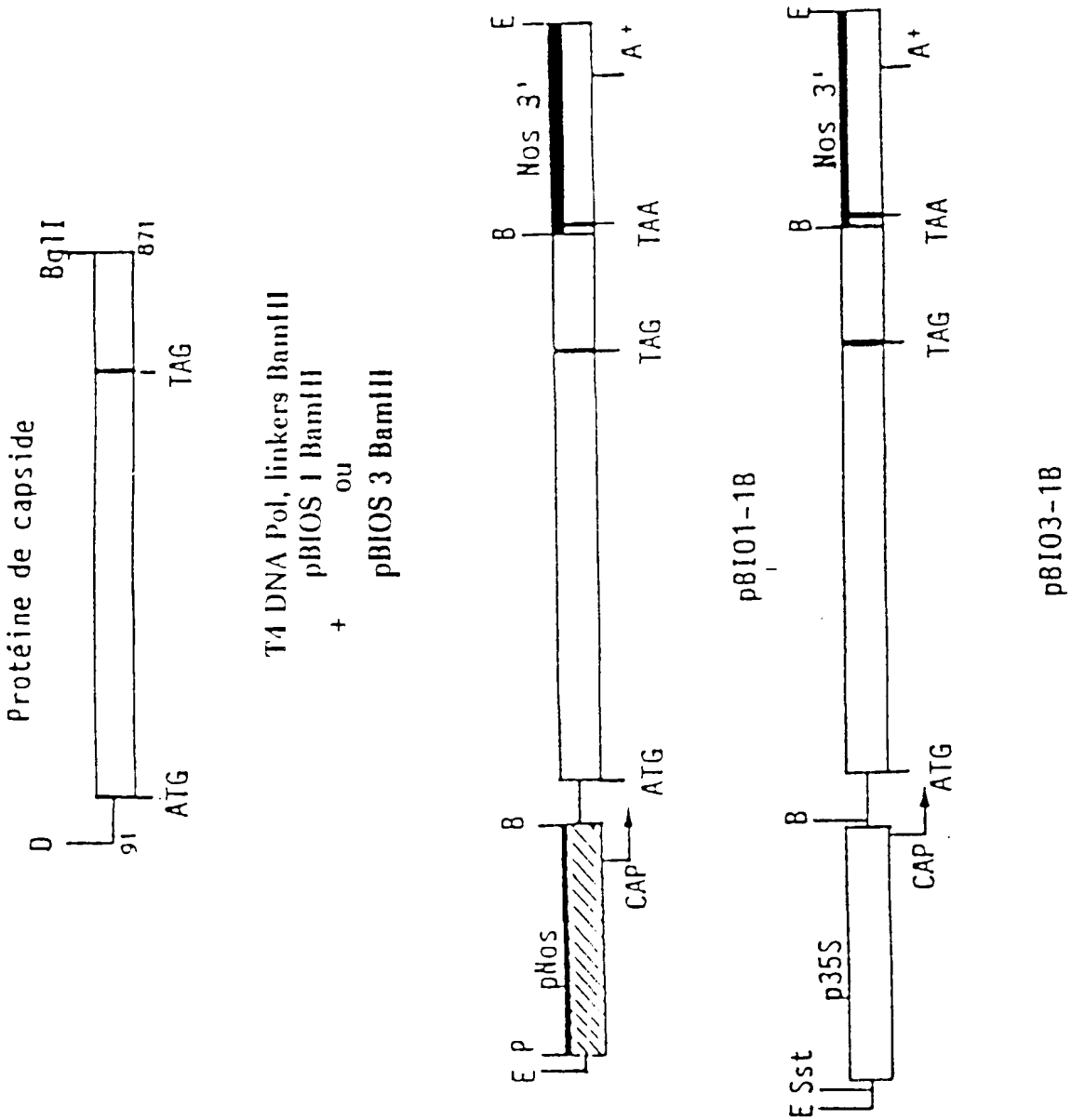


FIGURE 3


Protéine de capside
BglII
871
TAG
ATG
D
91
T4 DNA Pol, linkers BamHI
pBIOS 1 BamHI
ou
pBIOS 3 BamHI
+
E
A+
B
Nos 3'
TAA
TAG
ATG
B
CAP
pNos
E P
pBI01-1B
pBI03-1B
p35S
E Sst

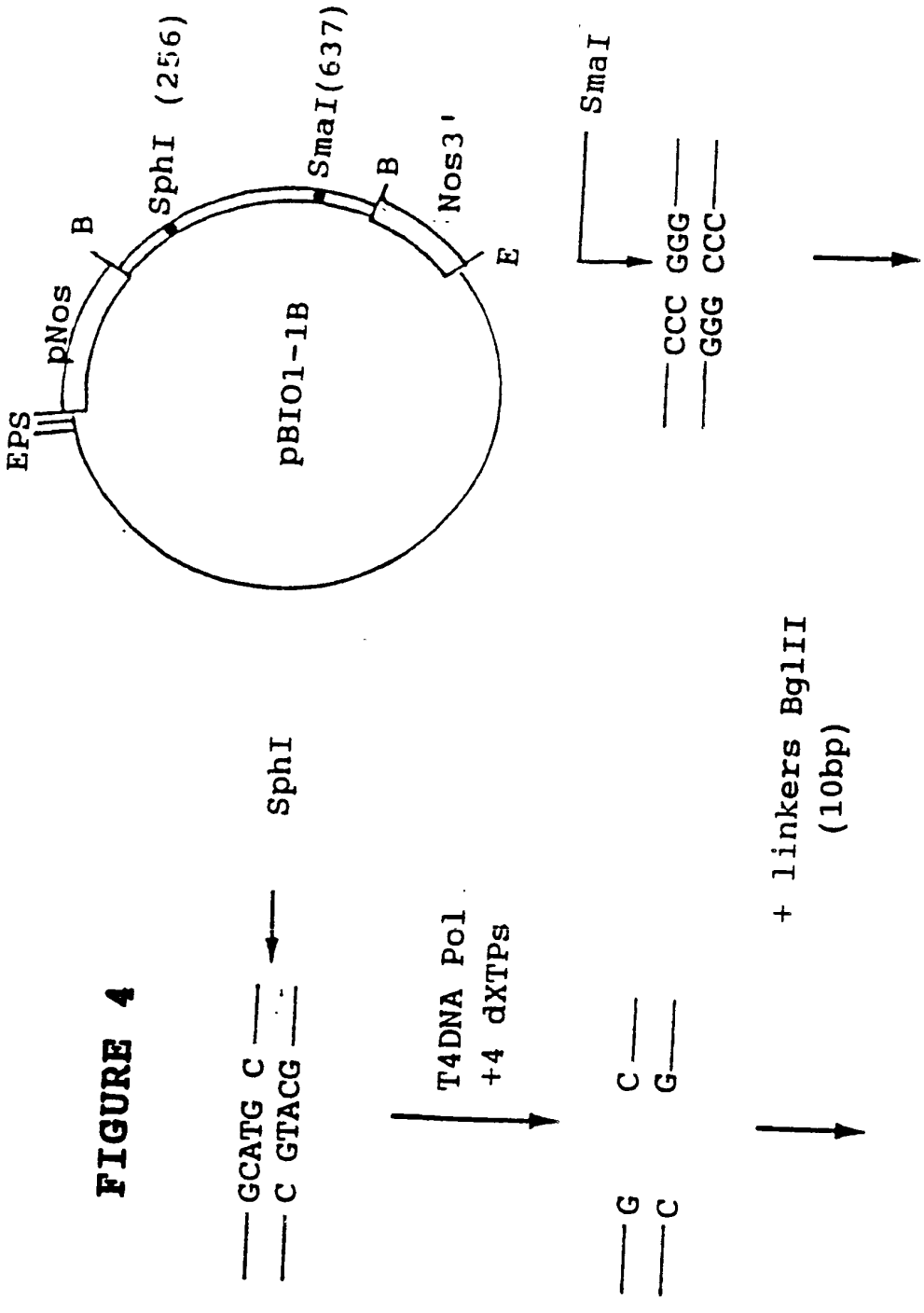
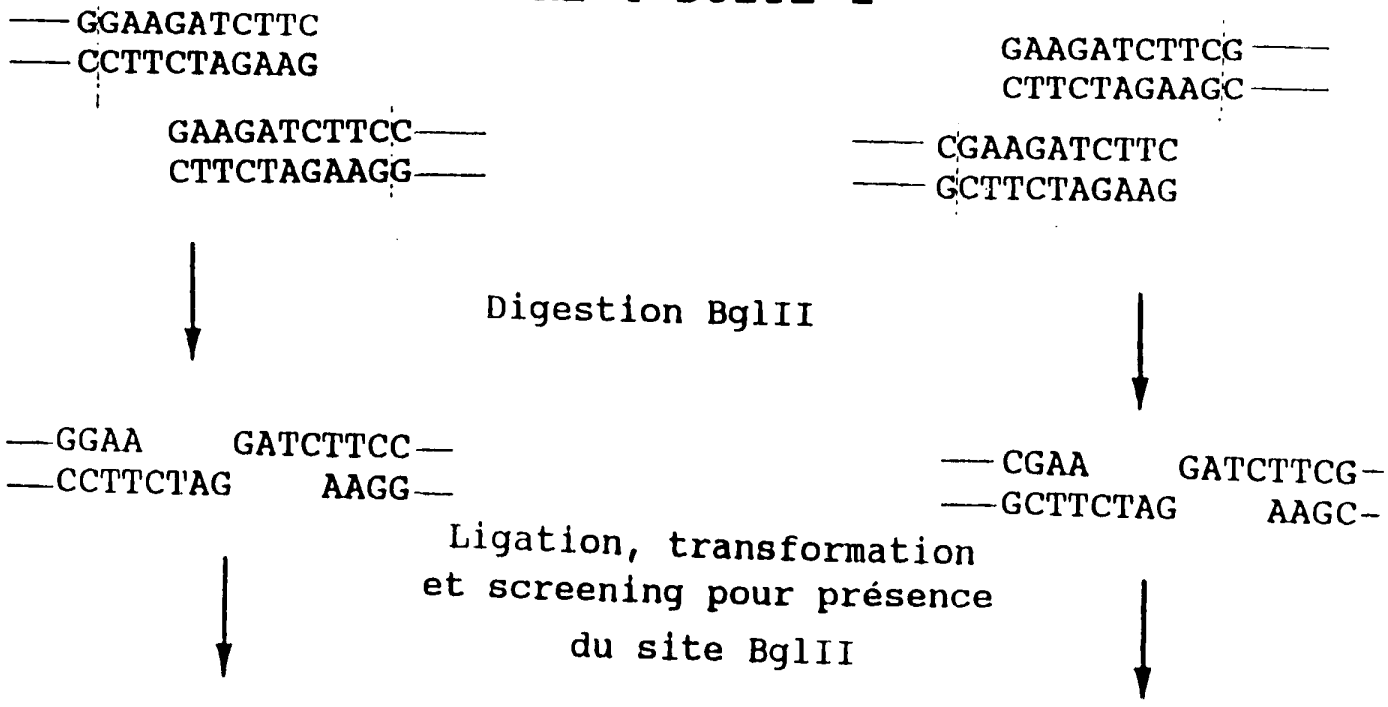


FIGURE 4

FIGURE 4 SUITE 1



36 37 38 39 40 41
TCG GAA GAT CTT CCT ATG

SER GLU ASP LEU PRO MET

Séquence codante mutageneisée

22K + 2A.a. et
changement de 2A.a.

164 165 166 167 168 169
ACC CGA AGA TCT TCG GGA TAA

THR ARG ARG SER SER GLY *

Séquence codante mutée

22K raccourci (-19A.a.) avec
changement des 4 derniers

FIGURE 4 SUITE 2

```
      36   37   38   39                                  164 165 166 167 168 169 170
--- TCG  CAT  GCT  ATG ----                          --  ACC CGG GAT AAA TTT GAG GAC --

      SER  HIS  ALA  MET                                 THR ARG ASP LYS PHE GLU ASP
```

6/43

22K Wild Type

7/13

FIGURE 5

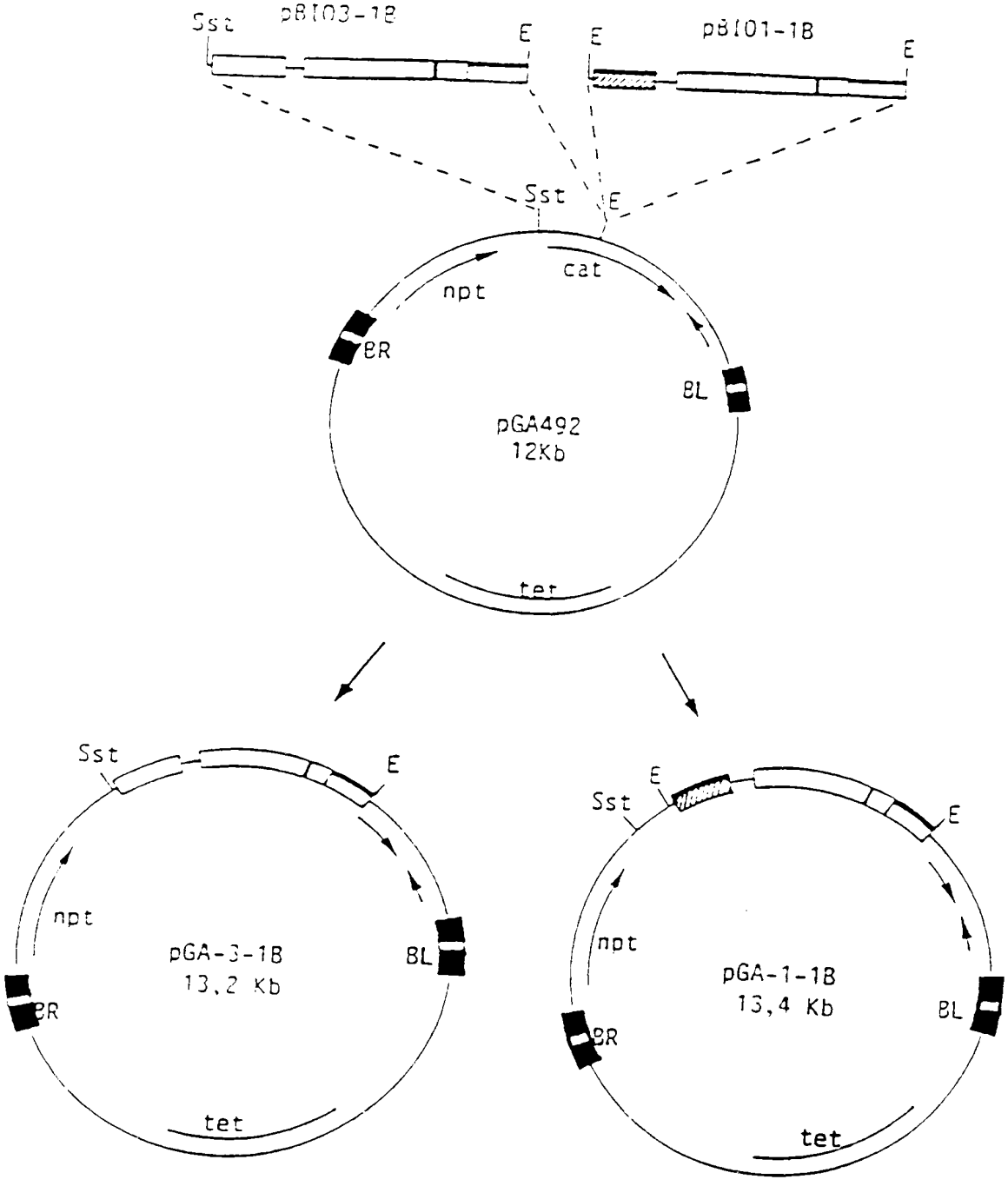

Sst
pBI03-1B
E
E
pBI01-1B
E
Sst
E
cat
npt
BR
BL
pGA492
12Kb
tet
Sst
E
npt
pGA-3-1B
13,2 Kb
BL
BR
tet
Sst
E
E
npt
pGA-1-1B
13,4 Kb
BL
BR
tet

FIGURE 6

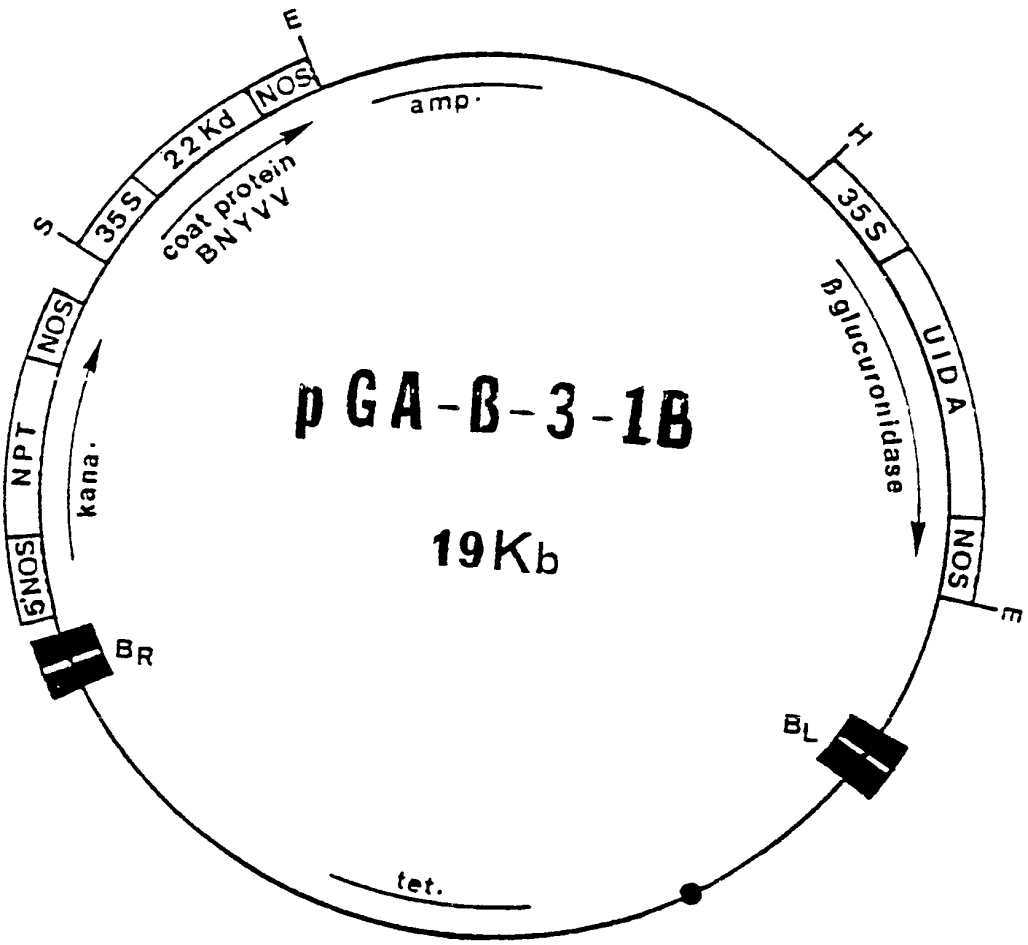


FIGURE 7

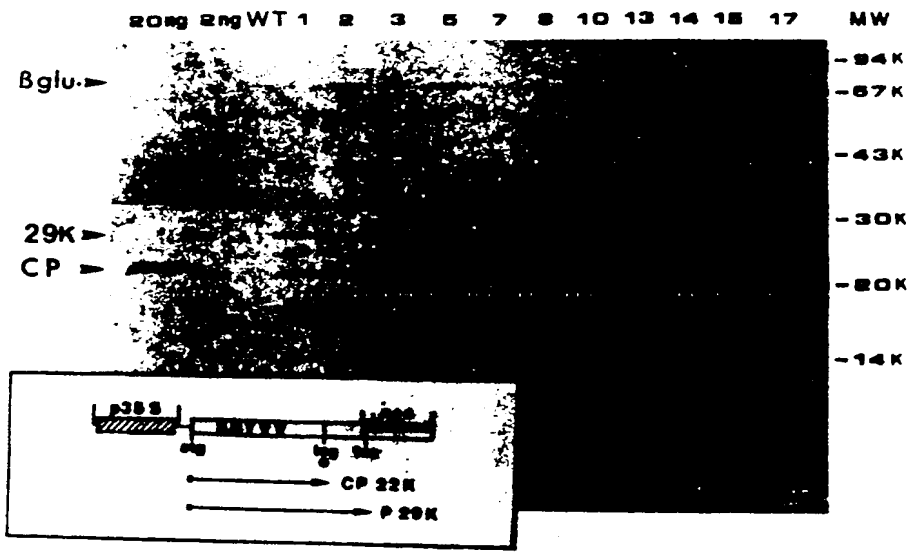

20mg
2mg
WT
1
2
3
5
7
8
10
13
14
15
17
MW
βglu.
29K
CP
94K
67K
43K
30K
20K
14K
35S
CP 22K
P 29K

FIGURE 8

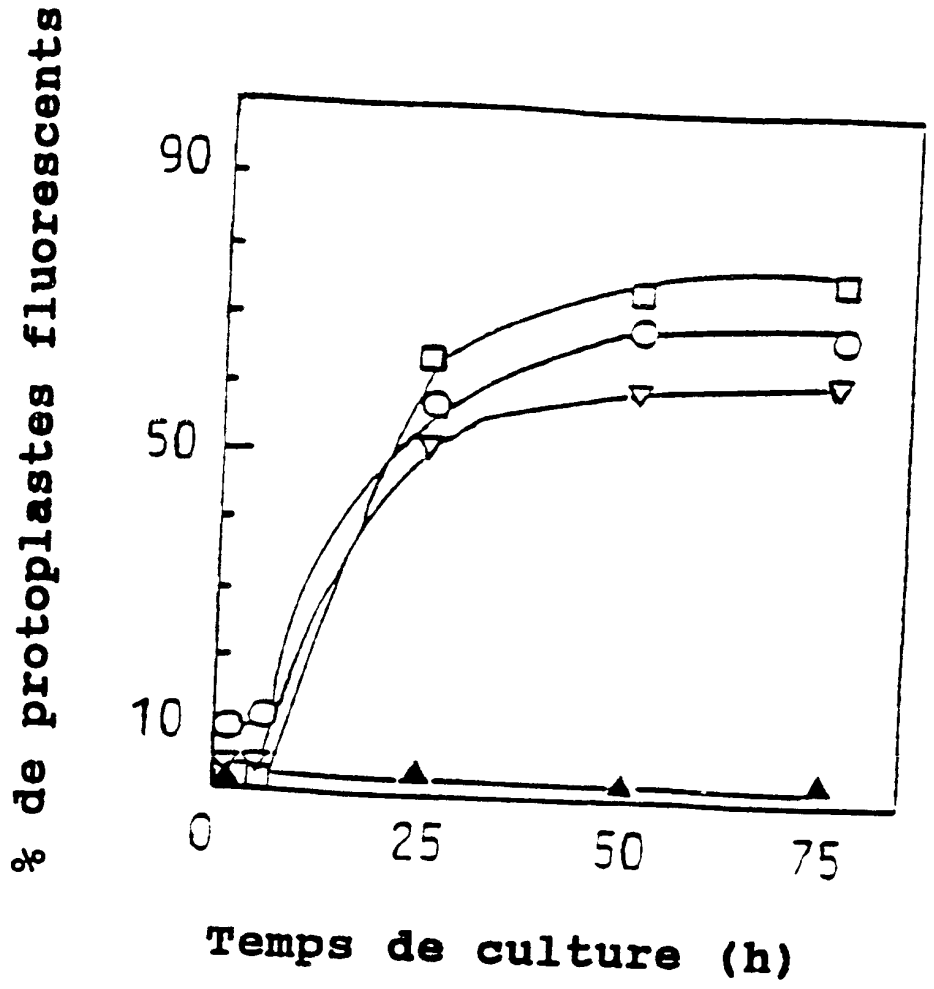


FIGURE 9

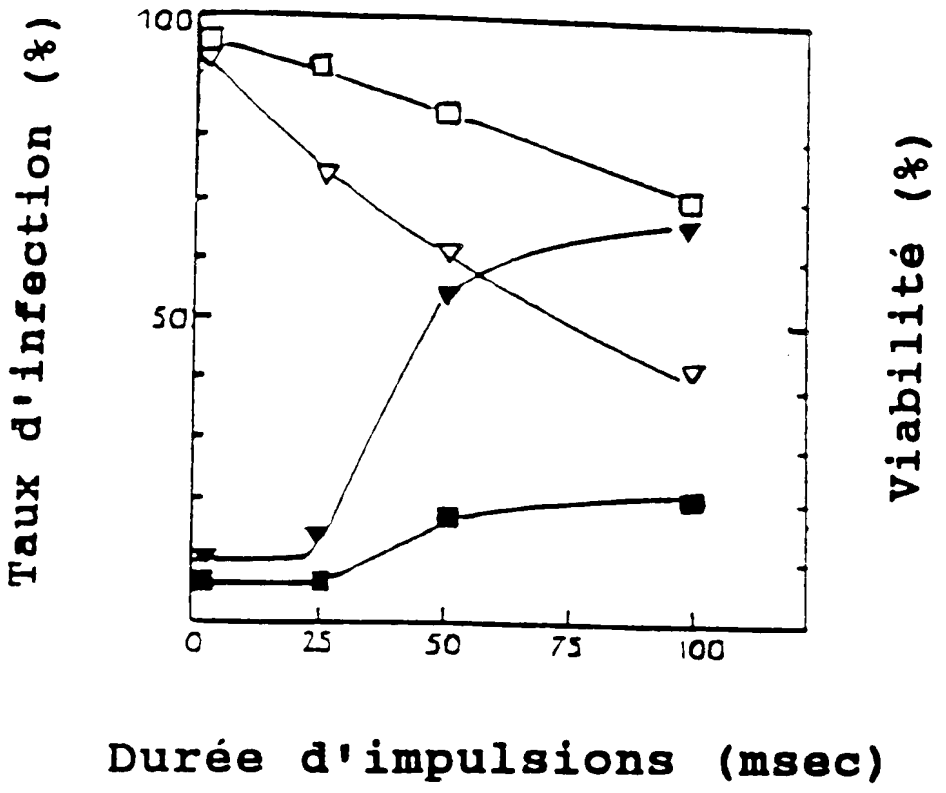
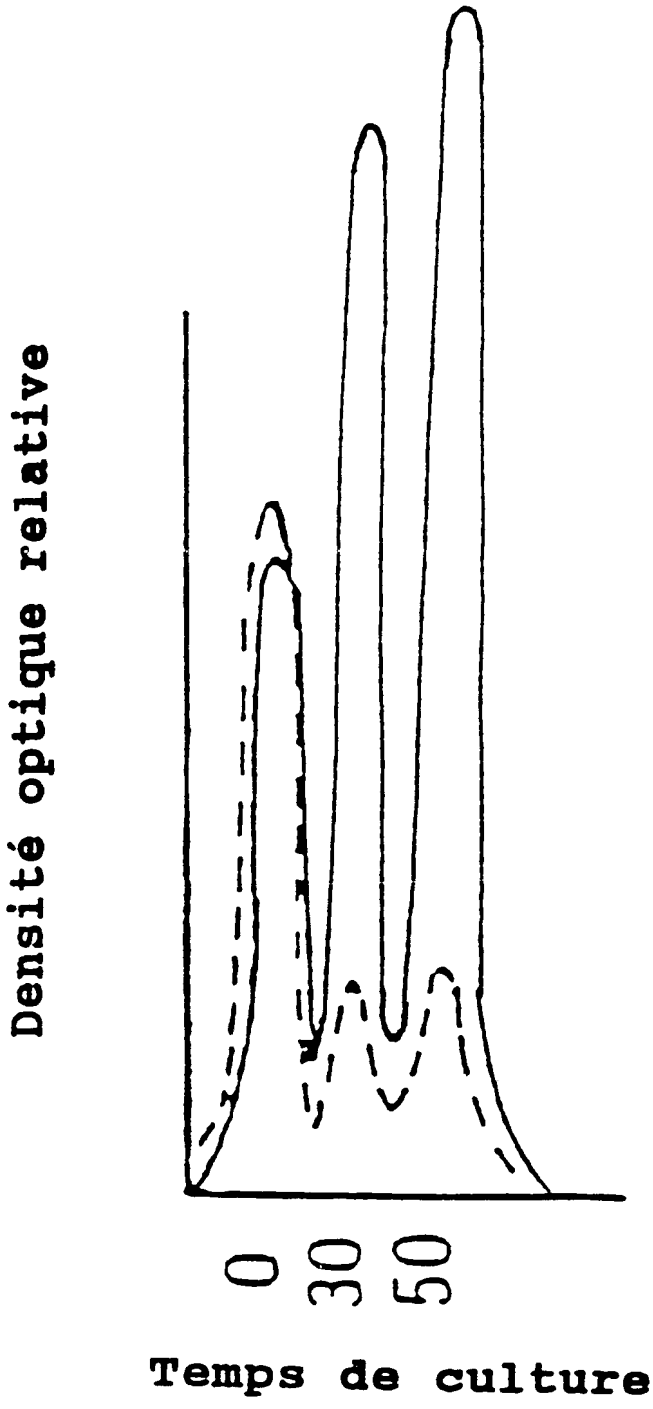

Taux d'infection (%)
100
50
0
25
50
75
100
Viabilité (%)
Durée d'impulsions (msec)

FIGURE 10



13/13

FIGURE 11